Selective DNA Binding and Association with the CREB Binding Protein Coactivator Contribute to Differential Activation of Alpha/Beta Interferon Genes by Interferon Regulatory Factors 3 and 7

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Recent studies implicate the interferon (IFN) regulatory factors (IRF) IRF-3 and IRF-7 as key activators of the alpha/beta IFN (IFN-α/β) genes as well as the RANTES chemokine gene. Using coexpression analysis, the human IFNB, IFNA1, and RANTES promoters were stimulated by IRF-3 coexpression, whereas the IFNA4, IFNA7, and IFNA14 promoters were preferentially induced by IRF-7 only. Chimeric proteins containing combinations of different IRF-7 and IRF-3 domains were also tested, and the results provided evidence of distinct DNA binding properties of IRF-3 and IRF-7, as well as a preferential association of IRF-3 with the CREB binding protein (CBP) coactivator. Interestingly, some of these fusion proteins led to supraphysiologic levels of IFN promoter activation. DNA binding site selection studies demonstrated that IRF-3 and IRF-7 bound to the 5'-GAAANN2GAAN-3' consensus motif found in many virus-inducible genes; however, a single nucleotide substitution in either of the GAAA half-site motifs eliminated IRF-3 binding and transactivation activity. These studies demonstrate that IRF-3 possesses a restricted DNA binding site specificity and interacts with CBP, whereas IRF-7 has a broader DNA binding specificity that contributes to its capacity to stimulate delayed-type IFN gene expression. These results provide an explanation for the differential regulation of IFN-α/β gene expression by IRF-3 and IRF-7 and suggest that these factors have complementary rather than redundant roles in the activation of the IFN-α/β genes.

Interferons (IFNs) are multifunctional secreted proteins involved in antiviral defense, cell growth regulation, and immune activation (44). Alpha/beta IFN (IFN-α/β) is produced by virus-infected host cells and constitutes the primary response against virus infection, while gamma IFN (IFN-γ), a TH1 cytokine produced by activated T cells and natural killer cells, is crucial in eliciting the proper immune response and pathogen clearance. Virus infection induces the transcription and synthesis of multiple IFN genes (16, 33, 44); newly synthesized IFN interacts with neighboring cells through cell surface receptors and the Janus-activated kinase (JAK)–STAT signaling pathway, resulting in the induction of over 30 new cellular proteins that mediate the diverse functions of the IFNs (6, 18, 21, 39).

Among the many virus- and IFN-inducible proteins are members of the growing family of interferon regulatory factors (IRFs), which now consists of nine members, as well as several virus-encoded IRFs (4). The presence of IRF-like binding sites in the promoter regions of the IFNB and IFNA genes implicated the IRFs as direct regulators of IFN-α/β gene induction (11–14, 29). Within the IRF family, IRF-3 and IRF-7 have recently been identified as key regulators of the induction of IFNs (reviewed in reference 26).

IRF-3 is expressed constitutively in a variety of tissues and demonstrates a unique response to virus infection (1). Latent cytoplasmic IRF-3 is posttranslationally modified and activated through phosphorylation of specific serine residues located in its C-terminal end following virus infection or treatment with double-stranded RNA (24, 45–47). Overexpression of IRF-3 significantly enhances virus-mediated expression of IFN-α/β genes and results in the induction of an antiviral state (19). Other studies have demonstrated that transcription of the CC-chemokine RANTES is upregulated by virus infection, mediated through IRF-3 activation and binding to overlapping ISRE-like elements in the −100 region of the RANTES promoter (23).

Structure-function analysis has revealed that IRF-3 contains an N-terminal DNA binding domain (DBD); a strong but atypical transactivation domain, located between amino acids 134 and 394, a region that also contains a nuclear export sequence element; a proline-rich region; and an IRF association domain (IAD). Two autoinhibitory domains in IRF-3 form an intramolecular interaction that results in a closed conformation and masks the IAD and the DBD to prevent nuclear translocation and subsequent DNA binding (25). Following virus infection, inducible phosphorylation of IRF-3 at the carboxy terminus relieves the intramolecular association between the two autoinhibitory domains, unmasking the IAD and the DBD. The conformational change in IRF-3 results in the formation of homodimers through the IAD. IRF-3 dimerization leads to cytoplasmic to nuclear translocation, association with the CREB binding protein (CBP) coactivator, and stimulation of DNA binding and transcriptional activities (reviewed in references 17 and 26). IRF-3 phosphorylation ultimately results in its degradation via the ubiquitin-proteasome pathway (24, 34). These biological features implicate IRF-3 as an important component of the immediate-early response to virus infection (17, 26).

IRF-7 was first described to bind and repress the Qp promoter region of the Epstein-Barr virus (EBV) EBNA-1 gene,
which contains an ISRE-like element (31, 48). Unlike IRF-3, IRF-7 is not expressed constitutively in cells; rather, expression is induced by IFN, lipopolysaccharide, and virus infection. As with IRF-3, virus infection appears to induce the phosphorylation of IRF-7 at its carboxy terminus, a region that is highly homologous to the IRF-3 C-terminal end (27, 37). IRF-7 also localizes to the cytoplasm in uninfected cells and translocates to the nucleus after phosphorylation (2, 37). Two groups have identified potential serine residues targeted for inducible phosphorylation by analogy to IRF-3. Marie et al. mutated Ser245 and Ser269 in the kinase domain of IRF-7, based on homology, to Ser385 and Ser386 in IRF-3. The mutant was not phosphorylated and did not activate IFN-α gene expression (27). Sato et al. generated a deletion mutant in which the region containing the potential sites of inducible phosphorylation between amino acids 411 to 453 was truncated. The mutant no longer translocated to the nucleus following virus infection, implicating inducible phosphorylation as a critical step for translocation (37).

Because of the common and distinct biological features of IRF-3 and IRF-7, we sought to identify the molecular basis for the differential activation of IFN-α/β genes by IRF-3 and IRF-7 in response to virus infection. Our results indicate that the distinct DNA binding specificities of IRF-3 and IRF-7, together with the different capacities of the IRF-3 and IRF-7 C-terminal domains to bind the CBP coactivator—provide an explanation for the differential regulation of IFN-α/β gene expression by these two transcription factors.

MATERIALS AND METHODS

Plasmid constructions and mutagenesis. Plasmids expressing the wild-type and mutated forms of IRF-3 were described previously (23–25). IRF-3 expression plasmids were prepared by cloning the IRF-7A cDNA (PCR amplified from pcDNA-IRF-7A; a gift from L. Zhang and J. Pagano) into the pFlag-CMV-2 vector to generate pFlag-IRF-7. pFlag-IRF-7 was digested with BglII and SalI and subcloned into the pGL3-Basic vector (Promega). The RANTES-pGL3 luciferase reporter was prepared by cloning the BglII/SalI fragment (from the RANTES-CAT reporter plasmid (25) into the pGL3-Basic vector (Promega)). The IRF-3(4E)/pGL3 (P10E/Q15E/N28E/K29E), IRF-3(5E)/pGL3 (P10E/Q15E/K29E/N30E/N31E), IRF-3(5E)/pGL3 (P10E/Q15E/L28E/K29E/N31E), IRF-3(5E)/pGL3 (P10E/Q15E/L28E/K29E/N30E), IRF-3(5E)/pGL3 (P10E/Q15E/L28E/K29E/N30E/N31E), IRF-3(5E)/pGL3 (P10E/Q15E/L28E/K29E/N30E/N31E), and IRF-3(5E)/pGL3 (P10E/Q15E/L28E/K29E/N30E/N31E) deleted mutant in which the region containing the potential sites of inducible phosphorylation between amino acids 411 to 453 was truncated. The mutant no longer translocated to the nucleus following virus infection, implicating inducible phosphorylation as a critical step for translocation (37).

Because of the common and distinct biological features of IRF-3 and IRF-7, we sought to identify the molecular basis for the differential activation of IFN-α/β genes by IRF-3 and IRF-7 in response to virus infection. Our results indicate that the distinct DNA binding specificities of IRF-3 and IRF-7, together with the different capacities of the IRF-3 and IRF-7 C-terminal domains to bind the CBP coactivator—provide an explanation for the differential regulation of IFN-α/β gene expression by these two transcription factors.

Immunoprecipitation and immunoblot analysis of protein–protein interactions. 293 cells were cotransfected with expression plasmids encoding wild-type or mutated forms of IRF-3 or IRF-7. Whole-cell extracts (200 to 500 μg) were prepared from cotransfected cells and incubated with 2 μl of anti-myc antibody 9E10, anti-IRF-3 antibody A22, or anti-IRF-7 antibody N-15, anti-PAF-250 antibody 6B3, or anti-PCAF antibody (a gift from X. Yang) cross-linked to 30 μl of protein A-Sepharose beads for 1 h at 4°C. Precipitates were washed five times with lysis buffer (23) and eluted by boiling the beads for 3 min in SDS sample buffer. Eluted proteins were separated by SDS-PAGE, transferred to a Hybond-N+ transfer membrane, and incubated with anti-Flag, anti-β-actin, or anti-calcium antibody (Amersham Corp.) at a dilution of 1:2,500. The reaction was then visualized with an enhanced chemiluminescence detection system as recommended by the manufacturer (Amersham).

Electromobility shift assay (EMSA). Whole-cell extracts were prepared 48 h after transfection with 5 μg of each expression plasmid. Cells were lysed in 10 mM Tris-Cl (pH 8.0)–60 mM KC1–1 mM EDTA–1 mM DTT–0.5% NP-40–0.5% 1 mM sodium dodecyl sulfate (SDS)–100 mM sodium acetate, 50 mM Tris-Cl (pH 7.6). The DNA was then recovered by ethanol precipitation. The recovered DNA was amplified by PCR using 100 pmol of forward primer and 100 pmol of reverse primer for 15 cycles under the conditions described above. After five rounds of selection, the protein-DNA complexes were separated by electrophoresis with a 5% polyacrylamide gel in 0.5× Tris-borate-EDTA (TBE). The bound DNA was excised from the dried gel and eluted in 400 μl of elution buffer 2 (0.5 M ammonium acetate, 1 mM EDTA [pH 8.0]). Bound DNA was recovered by ethanol precipitation and amplified by PCR. The products were then digested with EcoRI and BamHI cloning into pBlueScript KS+(+) and subjected to sequence analysis.

Immunoblot analysis. To confirm the expression of the transgenes, equivalent amounts of whole-cell extract (20 μg) were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) with a 10% polyacrylamide gel. After electrophoresis, proteins were transferred to a Hybond transfer membrane (Amersham) in a buffer containing 30 mM Tris, 200 mM glycine, and 200 μl of methanol for 1 h. The membrane was blocked in phosphate-buffered saline (PBS) containing 5% dried milk for 1 h and then probed with anti-Flag antibody M2 (Sigma) in 5% milk–PBS at a dilution of 1:3,000. These incubations were done at 4°C overnight or at room temperature for 1 to 3 h. After four 15-min washes with PBS, the membrane was reacted with a peroxidase-conjugated secondary goat anti-mouse antibody (Amersham Corp.) at a dilution of 1:2,500. The reaction was then visualized with an enhanced chemiluminescence detection system as recommended by the manufacturer (Amersham).
RESULTS

Differential induction of IFNA and IFNB promoters by IRF-3 and IRF-7. The IRF-7 transcription factor shares many structural features with IRF-3, including a structurally conserved DBD and a serine-rich C-terminal region that is the target of virus-inducible phosphorylation (27, 37). Because of the involvement of IRF-3 in the activation of immediate-early IFNB and IFNA genes and the role of IRF-7 in the induction of delayed-type IFNA genes (27), we compared the activation of IFN-α/β and RANTES promoters by different forms of IRF-3 and IRF-7. During studies on the structure and function of IRF-7, two constitutively active forms of IRF-7 were generated (R. Lin, Y. Mamane, and J. Hiscott, submitted for publication), one with a substitution of Ser477 and Ser479 with the phosphomimetic Asp [IRF-7(D477/479)] and the other with a deletion of a portion of the C-terminal domain [IRF-7(Δ247–467)]. Plasmids expressing wild-type IRF-3, IRF-3(5D), IRF-7, IRF-7(D477/479), IRF-7(Δ247–467), IRF-3/7, or IRF-7/3, as indicated. Luciferase activity was analyzed at 24 h posttransfection by the dual-luciferase reporter assay as described by the manufacturer (Promega). Relative luciferase activity was measured as fold activation (relative to the basal level for the reporter gene in the presence of the pFlag-CMV-2 vector after normalization to cotransfected relative light unit activity); the values represent the average of three experiments performed in duplicate, with variability of 10 to 25%.

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<td>IRF-7(Δ247–467)</td>
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pFlag-CMV-2 (vector alone)

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FIG. 1. Activities of IRF-7 and IRF-3 fusion proteins. The structures of the fusion proteins are illustrated schematically (Pro, proline-rich domain; TAD, transactivation domain; SRD, signal response domain; CAD, constitutive activation domain; ID, inhibitory domain). IRF-7/3A contains 541 amino acids, 246 from IRF-7 (1 to 246) and 295 from IRF-3(5D) (133 to 427). IRF-7/3B consists of 495 amino acids, 200 from IRF-7 (1 to 200) and 295 from IRF-3(5D) (133 to 427). IRF-7/3C consists of 445 amino acids, 150 from IRF-7 (1 to 150) and 295 from IRF-3(5D) (133 to 427). IRF-3/7 contains 265 amino acids, 132 from IRF-3 and 133 from IRF-7(D247–467). For transactivation assays, 293 cells were transfected with the pRLTK control plasmid, the RANTES-pGL3, IFNB-pGL3, IFNA1-pGL3, or IFNA4-pGL3 reporter plasmid, and the expression plasmids encoding IRF-3, IRF-3(5D), IRF-7, IRF-7(D477/479), IRF-7(Δ247–467), IRF-3/7, or IRF-7/3, as indicated. Luciferase activity was measured at 24 h posttransfection by the dual-luciferase reporter assay as described by the manufacturer (Promega). Relative luciferase activity was measured as fold activation (relative to the basal level for the reporter gene in the presence of the pFlag-CMV-2 vector after normalization to cotransfected relative light unit activity); the values represent the average of three experiments performed in duplicate, with variability of 10 to 25%.

Chimeric IRF-7 and IRF-3 proteins produce supraphysiological induction of RANTES and IFN promoters. The pos-
Transfection, cells were infected with Sendai virus for 6 h with various Flag-tagged IRF-7 or IRF-7/3 expression plasmids. At 24 h post-infection, the cells were lysed and subjected to immunoblotting (IB) with anti-Flag antibody. F7(2D), F7 D477/479.

**Figure 2.** DNA binding activity of IRF-7/3 chimeric proteins. (A) An EMSA was performed with whole-cell extracts (20 μg) derived from 293 cells transfected with various Flag-tagged IRF-7 or IRF-7/3 expression plasmids. At 24 h post-transfection, cells were infected with Sendai virus for 6 h (+) or left uninfected (−), as indicated. The 32P-labeled probe corresponded to the PRDII-PRDIII (5′-GAAAACCTGAAAGGGAGAAGTGAAAGTG-3′) motif of the IFNB promoter. (B) Twenty micrograms of whole-cell extracts from panel A was analyzed by immunoblotting (IB) with anti-Flag antibody. F7(2D), F7 D477/479.

**Table 1.** Summary of DNA binding activity of IRF-7/3 chimeric proteins.

<table>
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<tr>
<td>IRF-7</td>
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<tr>
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<td>-</td>
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<td>IRF-7/3B</td>
<td>-</td>
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<tr>
<td>IRF-7/3C</td>
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</table>

**Figure 2B.** DNA binding activity of IRF-7/3 chimeric proteins. (A) An EMSA was performed with whole-cell extracts (20 μg) derived from 293 cells transfected with various Flag-tagged IRF-7 or IRF-7/3 expression plasmids. At 24 h post-transfection, cells were infected with Sendai virus for 6 h (+) or left uninfected (−), as indicated. The 32P-labeled probe corresponded to the PRDII-PRDIII (5′-GAAAACCTGAAAGGGAGAAGTGAAAGTG-3′) motif of the IFNB promoter. (B) Twenty micrograms of whole-cell extracts from panel A was analyzed by immunoblotting (IB) with anti-Flag antibody. F7(2D), F7 D477/479.

**Figure 2C.** DNA binding activity of IRF-7/3 chimeric proteins. (A) An EMSA was performed with whole-cell extracts (20 μg) derived from 293 cells transfected with various Flag-tagged IRF-7 or IRF-7/3 expression plasmids. At 24 h post-transfection, cells were infected with Sendai virus for 6 h (+) or left uninfected (−), as indicated. The 32P-labeled probe corresponded to the PRDII-PRDIII (5′-GAAAACCTGAAAGGGAGAAGTGAAAGTG-3′) motif of the IFNB promoter. (B) Twenty micrograms of whole-cell extracts from panel A was analyzed by immunoblotting (IB) with anti-Flag antibody. F7(2D), F7 D477/479.

**Figure 2D.** DNA binding activity of IRF-7/3 chimeric proteins. (A) An EMSA was performed with whole-cell extracts (20 μg) derived from 293 cells transfected with various Flag-tagged IRF-7 or IRF-7/3 expression plasmids. At 24 h post-transfection, cells were infected with Sendai virus for 6 h (+) or left uninfected (−), as indicated. The 32P-labeled probe corresponded to the PRDII-PRDIII (5′-GAAAACCTGAAAGGGAGAAGTGAAAGTG-3′) motif of the IFNB promoter. (B) Twenty micrograms of whole-cell extracts from panel A was analyzed by immunoblotting (IB) with anti-Flag antibody. F7(2D), F7 D477/479.

**Figure 2E.** DNA binding activity of IRF-7/3 chimeric proteins. (A) An EMSA was performed with whole-cell extracts (20 μg) derived from 293 cells transfected with various Flag-tagged IRF-7 or IRF-7/3 expression plasmids. At 24 h post-transfection, cells were infected with Sendai virus for 6 h (+) or left uninfected (−), as indicated. The 32P-labeled probe corresponded to the PRDII-PRDIII (5′-GAAAACCTGAAAGGGAGAAGTGAAAGTG-3′) motif of the IFNB promoter. (B) Twenty micrograms of whole-cell extracts from panel A was analyzed by immunoblotting (IB) with anti-Flag antibody. F7(2D), F7 D477/479.

**Figure 2F.** DNA binding activity of IRF-7/3 chimeric proteins. (A) An EMSA was performed with whole-cell extracts (20 μg) derived from 293 cells transfected with various Flag-tagged IRF-7 or IRF-7/3 expression plasmids. At 24 h post-transfection, cells were infected with Sendai virus for 6 h (+) or left uninfected (−), as indicated. The 32P-labeled probe corresponded to the PRDII-PRDIII (5′-GAAAACCTGAAAGGGAGAAGTGAAAGTG-3′) motif of the IFNB promoter. (B) Twenty micrograms of whole-cell extracts from panel A was analyzed by immunoblotting (IB) with anti-Flag antibody. F7(2D), F7 D477/479.

**Figure 2G.** DNA binding activity of IRF-7/3 chimeric proteins. (A) An EMSA was performed with whole-cell extracts (20 μg) derived from 293 cells transfected with various Flag-tagged IRF-7 or IRF-7/3 expression plasmids. At 24 h post-transfection, cells were infected with Sendai virus for 6 h (+) or left uninfected (−), as indicated. The 32P-labeled probe corresponded to the PRDII-PRDIII (5′-GAAAACCTGAAAGGGAGAAGTGAAAGTG-3′) motif of the IFNB promoter. (B) Twenty micrograms of whole-cell extracts from panel A was analyzed by immunoblotting (IB) with anti-Flag antibody. F7(2D), F7 D477/479.

**Figure 2H.** DNA binding activity of IRF-7/3 chimeric proteins. (A) An EMSA was performed with whole-cell extracts (20 μg) derived from 293 cells transfected with various Flag-tagged IRF-7 or IRF-7/3 expression plasmids. At 24 h post-transfection, cells were infected with Sendai virus for 6 h (+) or left uninfected (−), as indicated. The 32P-labeled probe corresponded to the PRDII-PRDIII (5′-GAAAACCTGAAAGGGAGAAGTGAAAGTG-3′) motif of the IFNB promoter. (B) Twenty micrograms of whole-cell extracts from panel A was analyzed by immunoblotting (IB) with anti-Flag antibody. F7(2D), F7 D477/479.
recombinant IRF-3–GST or IRF-7–GST; the IRF-bound oligonucleotides were eluted, PCR amplified, and used as input for subsequent rounds of selection and purification. The PCR-amplified oligonucleotides recovered after each round of selection were subjected to gel shift analysis using recombinant IRF-3–GST (Fig. 5A) or IRF-7–GST (Fig. 5B). Oligonucleotides selected with IRF-3 consisted of enriched sequences that bound to IRF-3 (Fig. 5A, lanes 1 to 5) but interacted weakly with IRF-7 (Fig. 5A, lanes 6 to 10), while oligonucleotides selected with IRF-7 bound weakly to both IRF-7 (Fig. 5B, lanes 6 to 10) and IRF-3 (Fig. 5B, lanes 1 to 5). After five rounds of selection, bound oligonucleotides were purified from the gel, PCR amplified, and prepared for sequencing. The sequences recovered from 16 cloned IRF-3 binding sites and the consensus sequence are shown in Fig. 6. The sequences selected with IRF-3 were virtually identical to the consensus sequence recognized by IRF-1 and IRF-2 (5'-GAAANNGAAANN-3') (41).

Interestingly, the sequences recovered from 28 cloned IRF-7 binding sites were closely related to the IRF-3 consensus sequence, but upon closer examination it became clear that IRF-7 bound with greater flexibility than IRF-3 to the consensus binding site (Fig. 7). For example, from the sequences selected with IRF-3, 12 out of a total 16 sequences contained the tandem repeat GAAANNGAAANN motif, while from the sequences selected with IRF-7, only 5 of 28 sequences con-
FIG. 4. Binding of IRF-3 and IRF-7 to IRF binding sites of the IFN and RANTES promoters. (A) Recombinant N-terminal IRF-3 and IRF-7 bind to PRD-I-PRDIII and RANTES ISRE probes. An EMSA was performed with the indicated amounts of recombinant protein; the 32P-labeled probe corresponded to the PRD-I-PRDIII region (5'-GAAAACTGAAAGGGAGAAGTGAAAGTG-3') or the ISRE of the RANTES gene (5'-CTATTTCAGTTTTTCTTTTCCGTTTTGTG-3'). (B) Recombinant N-terminal IRF-3 and IRF-7 bind to PRD-I and PRD-III probes. An EMSA was performed with the indicated amounts of recombinant protein; the 32P-labeled probe corresponded to PRD-I (5'-GAGAAGTGAAAGTG-3') or PRD-III (5'-GAAAACTGAAAGGG-3'). (C) Recombinant N-terminal IRF-3 and IRF-7 bind to the PRD-I-like and TG sites from the IFNA1, IFNA2, and IFNA14 promoters. An EMSA was performed with the indicated amounts of IRF-3 or IRF-7 and 32P-labeled probes corresponding to the following PRD-I-like sites: IFNA1, 5'-GGAAAGCAAAAATGAAAGTGAGAAGTG-3'; IFNA2, 5'-GAAAAGCAAAAAGGAAGTGAAGAAGTG-3'; and IFNA14, 5'-GGAAAGGCAAAAAGGAAGTGAAGAAGTG-3'.

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tained the GAAANNGAAANN motif. The majority of the IRF-7 binding sites had a minimum of one nucleotide replacement in either the 5'-GAAA or the 3'-GAAA motif of the half-site. Thus, for IRF-7, either the 5' or the 3' half-site consisted of GAAANN and the other half-site contained at least one nucleotide substitution. This result indicates that a single nucleotide alteration in the GAAANNGAAANN consensus site precluded the binding of IRF-3 to ISRE or PRDI-like sites, whereas the nucleotide replacement(s) did not affect IRF-7 DNA binding.

**Binding of IRF-3 and IRF-7 to selected sequences.** The difference between the DNA binding sites selected with IRF-3 and IRF-7 is minimally a single nucleotide replacement in one of the GAAA core sequences of the GAAANNGAAANN motif. Oligonucleotides from the binding site selection were subjected to a DNA gel shift binding competition analysis to determine the relative binding affinities of IRF-3 and IRF-7 for different sites. IRF-3 binding to the GAAACCGAAACT oligonucleotide (Fig. 8A, a) was competed by the homologous GAAACCGAAACT motif (a) or the AAAACCGAAACT motif (e) (Fig. 8A, lanes 2 and 6) but not by oligonucleotide b, c, d, f, or g (Fig. 8A, lanes 3 to 5, 7, and 8), which were altered by one nucleotide in one of the GAAA core motifs. With IRF-7, all oligonucleotides (a to f) (Fig. 8A, lanes 10 to 15) were able to compete for the binding of IRF-7 to the GAAA CCGAAACT motif. The competition for binding was specific, since oligonucleotide g, which contained one intact GAAANN half-site, failed to compete for the binding of either IRF-3 or IRF-7 (Fig. 8A, lanes 8 and 16).

The ability of IRF-3 and IRF-7 to activate transcription from minimal TK promoter constructs linked to two copies of oligonucleotides a to f was next examined (Fig. 8B). Cotransfection of the constitutively active form of IRF-3 led to an 18- and 28-fold induction of reporter genes containing two copies of GAAACCGAAACT (Fig. 8B, a) and AAAACCGAAACT (e) motifs, respectively. However, no induction was observed from promoter constructs containing two copies of TAAACCGGA ACT, CAAACCCGAAACT, GAATTCGAAAGT, or GAAAG TGAACGC (Fig. 8B, b, c, d, and f). Interestingly, coexpression of IRF-7Δ247-467 stimulated expression from all reporter genes to different extents, consistent with the DNA binding competition analysis results.

**Differential gene activation by the IRF-like and TG motifs of IFNA promoters.** To determine whether the PRDI-like and TG sites (−98 to −71) of the different IFNA promoters were sufficient to mediate virus-induced or IRF-3- or IRF-7-activated expression, the same sequences that were used as probes in the EMSA were cloned upstream of a minimal TK promoter in the pGL3 luciferase reporter construct. As shown in Table 1, row 1, a single copy of PRDI-like and TG sites from the IFNA1 promoter was not responsive to Sendai virus infection and was only weakly activated by the constitutively active form of IRF-3 (5-fold); this element was moderately induced by coexpression of either IRF-7 (6-fold) or the constitutively active form of IRF-7 (Fig. 6).
(18-fold). Sendai virus infection together with IRF coexpression further augmented (two- to threefold) the induction of the PRDI-like and TG sites from the IFNA1 promoter. Two copies of the PRDI-like and TG sites from the IFNA1 promoter were generally more responsive to IRF-3 and IRF-7 stimulation (Table 1, row 2). In particular, IFNA1 was strongly activated by the constitutively active forms of IRF-3 and IRF-7 (300- and 270-fold induction, respectively). Two copies of the PRDI-like and TG sites from the IFNA1 promoter were also responsive to Sendai virus infection (12-fold induction). Coexpression of different forms of IRF-3 or IRF-7 generally had the effect of increasing the virus-induced activation of the IFNA1 promoter (Table 1, row 2).

The construct carrying a single copy of the PRDI-like and TG regions of IFNA2 was similarly weakly responsive to IRF coexpression and/or virus activation (Table 1, row 3). However, two copies of the PRDI-like and TG sites from the IFNA2 promoter were strongly activated by the constitutively active form of IRF-7 (120-fold induction) but were not activated by IRF-3(5D) (Table 1, row 4), consistent with the differential binding of IRF-7 and IRF-3 to the IFNA2 promoter (Fig. 4C). Two copies of the PRDI-like and TG sites from IFNA14 were activated modestly by IRF-7 (about 3- to 10-fold) but were not stimulated by IRF-3(5D) (Table 1, row 5). Even with four copies of the PRDI-like and TG sites from IFNA14, the activation of this promoter by IRF-7 and/or Sendai virus infection was in the range of 20- to 80-fold (Table 1, row 6). These results support the observations of Fig. 1 and 4 demonstrating that the PRDI-like and TG sites of the IFNA promoter are differentially regulated by the IRF-3 and IRF-7 transcription factors. The requirement for multimerization of these domains further suggests that these elements likely cooperate with other cis regulatory elements to control IFNA gene expression.

Primary sequence differences in the DBD of IRF-7 contribute to DNA binding affinity. The results from PCR-mediated DNA binding site selection indicated that IRF-3 binds to the consensus site recognized by IRF-1 and IRF-2 (5’-GAAA NNGAAAAN-3’ (41)), while IRF-7 binds with greater flexibility than IRF-3 to a related sequence (Fig. 6 and 7). In an attempt to identify amino acid differences that may contribute to differential DNA binding specificity, the amino acid sequences within the DBDs of IRF-1, IRF-2, IRF-3, and IRF-7 were compared by sequence alignment. Two major differences are present in IRF-7: (i) IRF-7 contains four acidic residues in the N-terminal region of the DBD (E16E21 in helix a1 and D34E35 in antiparallel β sheet b1), compared with the mutated or chimeric proteins were characterized by a decrease in DNA binding affinity of more than 100-fold compared with the affinity of wild-type IRF-3 (Fig. 9B, lanes 1 and 2) or IRF-7 (lanes 11 and 12). These results indicate that primary sequence differences in the DBD of IRF-7 contribute to differential DNA binding affinity and/or affinity to related sequences. However, all of the mutated or chimeric proteins were characterized by a decrease in DNA binding affinity of more than 100-fold compared with the affinity of wild-type IRF-3 (Fig. 9B, lanes 1 and 2) or IRF-7 (lanes 11 and 12). These results indicate that primary sequence information does not predict crucial amino acid residues involved in differential specificity. Rather, the intact three-dimensional structure of IRF-3 and IRF-7 is required to convey subtle differences in DNA binding specificity.

DISCUSSION

In the present study, we sought to examine the molecular basis for the differential regulation of several members of the IFN-α/β gene family (IFNA and IFNB) by IRF-3 and IRF-7. The IFNB, IFNA1, IFNA2, and RANTES promoters were activated by coexpression of either IRF-3 or IRF-7, whereas the IFNA4, IFNA7, and IFNA14 promoters were exclusively activated by IRF-7 and not by IRF-3. Analysis of protein-DNA interactions revealed that recombinant IRF-3 and IRF-7 selectively bound to different regions of the IFNB promoter; IRF-3 bound preferentially to the PRDIII domain of the IFNB promoter, while IRF-7 interacted exclusively with the PRDI domain. PCR-mediated DNA binding site selection results demonstrated that IRF-3 recognized the IRF consensus element 5’-GAAANNGAANNN-3’. Replacement of a single nucleotide within the GAAA core half-site was sufficient to preclude IRF-3 DNA binding. IRF-3 bound to a related sequence motif but with greater flexibility than IRF-3; a single nucleo-
tide replacement did not decrease IRF-7 DNA binding. These results demonstrate that the DNA binding site specificities of IRF-3 and IRF-7, together with the different capacities of the IRF-3 and IRF-7 C-terminal domains to bind the CBP coactivator, provide a partial explanation for the differential regulation of IFN-α/β gene expression by these two transcription factors.

The chimeric forms of IRF-7 and IRF-3 generated during the present study combined the DNA binding specificity of the IRF-7 DBD with the strong transactivation capacity of the IRF-3(5D) C-terminal domain. When tested with the IFNA and IFNB promoters, 10- to 20-fold-higher levels of reporter gene activity were observed with the chimeric proteins than with the constitutively active forms of either protein alone. Furthermore, the strong

FIG. 8. Characterization of selected binding sites. (A) An EMSA was performed with 20 ng of recombinant IRF-3–GST (lanes 1 to 8) or IRF-7–GST (lanes 9 to 16), 32P-labeled oligonucleotide a (5'-GAAACCGAAACTGAAACCGAAACT-3'), and a 1,000-fold molar excess of competitor DNA. Selected binding sites (two copies, indicated beside the gel as a to g) were used as competitors. (B) Activation of selected promoters by IRF-3 and IRF-7. 293 cells were transfected with the pRLTK control plasmid, reporter constructs containing the minimum TK-luciferase promoter and two copies of selected binding sites (designated a to f), and the active forms of IRF-3(5D) and IRF-7(Δ247–467) expression plasmids, and luciferase activity was analyzed at 24 h posttransfection. Relative luciferase activity was measured as fold activation (relative to the basal level for the reporter gene in the presence of the pFlag-CMV-2 vector after normalization to cotransfected relative light unit activity); the values represent the average of three experiments performed in duplicate, with variability of 10 to 25%.

A

<table>
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<th>Competitor</th>
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<td>IRF-7(150)/GST</td>
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| a: | GAAACCGAAACT |
| b: | GAAAGTGAACGC |
| c: | GAATTCGAAAGT |
| d: | TAAACCGAAGT |
| e: | AAAAAACGAAACT |
| f: | CAAACCGAAGT |
| g: | GAAAGTCTTCGC |

B

![Graph showing relative luciferase activity](https://example.com/graph.png)

- CMV-2
- IRF-3(5D)
- IRF-7(Δ247–467)
transcriptional activity of the IRF-7/3 chimera was not matched by the IRF-3/7 fusion protein. It is possible that the more restricted DNA binding specificity of the IRF-3 DBD and the apparent lack of interaction of IRF-7 with the histone acetyltransferase coactivators account for the difference between these chimeric forms. The failure of wild-type IRF-7, constitutively active IRF-7, or virus-activated IRF-7 to associate with CBP, p300, or PCAF was surprising, particularly since IRF-7 was able to stimulate IFN gene expression (Fig. 1). It is possible that IRF-7 interacts in a restricted manner with a distinct histone acetyltransferase coactivator or recruits coactivators only when bound to DNA and in association with other factors. The activity of IRF-7/3 in human cells stably transfected with IRF-7/3-expressing constructs has been difficult to characterize, since it appears that the expression of the chimeric protein is proapoptotic. Inducible regulation of the IRF-7/3-expressing constructs may provide an interesting system for examining downstream IFR-regulated genes.

TFIID has been identified as a potential target for transcriptional regulation (40). TFIID is a multimeric protein complex consisting of TBP and many TAFII18. Although TBP alone is able to bind core promoters containing TATA elements and can support basal transcription, TAFII18 are required for activated transcription. Some TAFII18 have been shown to serve as coactivators that directly contact enhancer-bound activators to modulate gene-specific transcription (43). TAFII250 (CCG1), a cell cycle regulatory protein thought to be important for progression through G1 phase, is one of the TAF subunits of TFIID (36). It binds directly to TBP as well as several other TAFs, including TAFII32 and TAFII70 (5). TAFII250 has also been shown to possess all histone acetyltransferase activity (30) and a protein kinase activity (7). The histone acetyltransferase activity of TAFII250 is conserved in yeasts, flies, and humans, and it may play an important role in controlling access of the transcription machinery to nucleosome-bound promoter sequences. TAFII250 is a bipartite protein kinase, consisting of N- and C-terminal kinase domains, and directly interacts with and phosphorylates RAP74, the large subunit of TFIIF (7, 35). The association of TAFII250 with IRF-3 and IRF-7 may target histone acetyltransferase to IRF target promoters and allow TFIID to gain access to transcriptionally repressed chromatin.

In virus-infected human and murine cells, IFNA and IFNA4 genes are coordinately induced, and their individual mRNAs are expressed at different levels. Human IFNA1, IFNA2, and IFNA4 are highly expressed in virus-infected peripheral blood mononuclear and lymphoblastoid Namalwa cells; their respective mRNA levels are 5- to 20-fold higher than those of IFNA5, IFNA7, IFNA8, and IFNA14 in the same cells (15). Since the virus-responsive elements within the IFNA gene promoters contain multiple GAAANN sequences similar to the PRDI and PRDIII domains of IFNB, many studies have shown that the PRDI-like sites of the human IFNA gene promoters are essential for IFNA-induced expression (reviewed in reference 3). In this study, we show that IRF-7 specifically binds to the PRDI site of the IFNB gene promoter. Activation of the IFNA gene promoters by IRF-3 and IRF-7 likewise occurs through the binding of IRF-7 to the PRDI-like sites of the IFNA gene promoters, based on the fact that recombinant IRF-7 binds to the PRDI-like sites of the IFNA1, IFNA2, and IFNA14 promoters.

Recent molecular and biological results have also suggested a temporal regulation of IFN gene activation by IRF-3 and IRF-7 (reviewed in reference 26). IFN-α/β genes can be subdivided into two groups: (i) immediate-early genes activated in response to virus infection by a protein synthesis-independent pathway (IFNB and murine IFNA4, which is equivalent to human IFNA1); and (ii) delayed-type genes (which include the other IFNA subtypes), whose expression is dependent on de novo protein synthesis (27). Following virus infection, IRF-3, NF-κB, and ATF-2/c-Jun are posttranslationally activated by inducer-mediated phosphorylation. These proteins cooperate to form a transcriptionally active enhancerosome at the IFNB promoter, together with the CBP transcriptional coactivator and the chromatin-associated high-mobility-group protein (9, 40). IRF-3 also upregulates murine IFNA4 expression (27). Secreted IFN produced from a subset of initially infected cells acts through an autocrine and paracrine loop which requires intact IFN receptors and JAK-STAT pathways. IFN activation of the IFN-stimulated gene factor 3 complex results in the transcriptional upregulation of IRF-7 (27, 37).

Virus infection activates IRF-7 through inducible phosphorylation, and phosphorylated IRF-7 participates together with IRF-3 in the transcriptional induction of immediate-early and delayed-type IFN genes (27, 37). In mice with a targeted disruption of either STAT-1, p48, or the IFN-β promoter, together with the CBP transcriptional coactivator and the chromatin-associated high-mobility-group protein (9, 40), IRF-3 is not upregulated but, rather, the amplification loop of IFN induction is not observed. Finally, the formation of distinct homo- or heterodimers between activated IRF-3 and activated IRF-7 may lead to differential regulation of target IFN genes (27, 37).

Recently, the crystal structure of the DBDs of IRF-1 and IRF-2 bound to DNA demonstrated that AANN(AAA) is the sequence physically recognized by IRF-1 and IRF-2 (8, 10). Our DNA binding site selection demonstrated that DNA sequences recognized by IRF-3 are identical to the IRF-E consensus element G(A)AANNGAAAAN, the consensus sequence for IRF-1 and IRF-2 (41). IRF-7 binds to related sequence elements but with greater flexibility in binding site specificity. From the sequences selected with IRF-3, 12 out of a total 16 sequences contained a tandem repeat of GAAA, while with IRF-7, only 5 out of 28 sequences contained the GAAANGAAANN consensus motif. Most of the sequences selected with IRF-7 had at least one nucleotide replacement in either the 5′-GAAA or the 3′-GAAA core motif. Furthermore, the oligonucleotides containing two copies of selected IRF-7 binding sites with one nucleotide replacement in either 5′-GAAA (GAATTCCGAAA) or 3′-GAAA (GAAGTTG AACGC) were able to compete for IRF-7 binding but not for...
IRF-3 binding to IRF-E. Consistent with their DNA binding activities, reporter constructs carrying two copies of these two binding sites were activated by a constitutively active form of IRF-7 but not by a constitutively active form of IRF-3.

As shown in Table 1, several PRDI-like sequences from different IFNA promoters were also analyzed for IRF-3 and/or IRF-7 transactivation. IRF-7 bound to the PRDI-like motif of the IFNA1, IFNA2, and IFNA14 promoters and activated transcription from the reporter gene promoters containing these motifs. In contrast, IRF-3 bound to the PRDI-like motif of IFNA1 and activated the expression of a reporter gene containing the PRDI-like motif from IFNA1 but did not bind to or activate related sequences from IFNA2 or IFNA14.

The flexibility in the binding site specificity of IRF-7 indicates that more target genes may be recognized by IRF-7 than by IRF-3; for example, genes containing the sequence GAA NNGAAANN (interleukin 4, HLA-B7, major histocompatibility complex class I, H-2D^d, immunoglobulin AB, 2',5'-oligoadenylate synthase, and Mx) or the sequence GAAATGGAAG (interleukin 7 receptor) may be preferentially activated by IRF-7 but not by IRF-3. The restricted binding site specificity of IRF-3 is nonetheless consistent with its role as a specific inducer of immediate-early IFN genes.

In conclusion, we have demonstrated that despite an overall similarity in structure between IRF-3 and IRF-7, both transcription factors possess unique functional characteristics and share complementary rather than redundant roles in the activation of the IFN-\(\alpha/\beta\) genes.
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