The Cell Cycle Control Element of Histone H4 Gene Transcription Is Maximally Responsive to Interferon Regulatory Factor Pairs IRF-1/IRF-3 and IRF-1/IRF-7*

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Interferon regulatory factors (IRFs) are transcriptional mediators of interferon-responsive signaling pathways that are involved in antiviral defense, immune response, and cell growth regulation. To investigate the role of IRF proteins in the regulation of histone H4 gene transcription, we compared the transcriptional contributions of IRF-1, IRF-2, IRF-3, and IRF-7 using transient transfection assays with H4 promoter/luciferase (Luc) reporter genes. These IRF proteins up-regulate reporter gene expression but IRF-1, IRF-3, and IRF-7 are more potent activators of the H4 promoter than IRF-2. Forced expression of different IRF combinations reveals that IRF-2 reduces IRF-1 or IRF-3-dependent activation, but does not affect IRF-7 function. Thus, IRF-2 may have a dual function in histone H4 gene transcription by acting as a weak activator at low dosage and a competitive inhibitor of other strongly activating IRFs at high levels. IRF-1/IRF-3 and IRF-1/IRF-7 pairs each mediate the highest levels of site II-dependent promoter activity and can up-regulate transcription by 120–150-fold. We also find that interferon γ up-regulates IRF-1 and site II-dependent promoter activity. This up-regulation is not observed when the IRF site is mutated or if cells are preloaded with IRF-1. Our results indicate that IRF-1, IRF-2, IRF-3, and IRF-7 can all regulate histone H4 gene expression. The pairwise utilization of distinct IRF factors provides a flexible transcriptional mechanism for integration of diverse growth-related signaling pathways.

INTERFERON REGULATORY FACTORS (IRFs) form a large family of transcription factors involved in antiviral defense, immune activation, and cell growth regulation. IRFs were initially identified as regulators of interferon genes in response to viral infection. However, it has subsequently been shown that there are at least nine cellular IRF proteins (IRF-1, IRF-2, IRF-3, IRF-4/Pip/ICSAT, IRF-5, IRF-6, IRF-7, ICSBP/IRF8, and ISGF3γ/p48/IRF9), as well as virally encoded forms, with broad biological functions (1, 2). All members of the IRF family share significant homology in the N-terminal 115 amino acids, which comprise the DNA-binding domain. For the IRF-3, IRF-4, IRF-5, IRF-8, and IRF-9 proteins, the homology extends into the C-terminal region with which these IRFs interact with other proteins or family members. Current data indicate that IRFs can function as transcriptional activators (e.g. IRF-1, IRF-3, and IRF-9), repressors (e.g. IRF-8), or both (e.g. IRF-2, IRF-4, and IRF-7). Studies with IRF-expressing cell lines and IRF knockout mice reveal that IRF family members have distinct roles in various biological processes, including cytokine signaling, responses to pathogens, cell growth regulation, and hematopoietic development (1–3).

IRF-1 and IRF-2 are transcription factors that interact with the same DNA sequence element (designated ISRE/IRF-E) in the promoters of type I interferon (IFN) and other cytokine inducible genes (4–8). IRF-1 is up-regulated by type I interferons and the type II interferon, IFN-γ (2). IRF-2 is up-regulated by IFN-1 and antagonizes IRF-1 activation by competing with IRF-1 for its DNA-binding site (4, 9–12). IRF-2 also functions as a transcriptional activator (13) and has been shown to activate the genes for histone H4 (14, 15), Epstein-Barr virus nuclear antigen-1 (EBNA-1) (16), and murine muscle vascular cell adhesion molecule-1 (17). In addition, IRF-1 and IRF-2 can co-occupy the Class II transactivator type IV promoter element IRF-E and synergistically activate this promoter (18).

IRF-1 and IRF-2 are key regulators of cell growth, cell cycle, and apoptosis, and function as an anti-oncogene and oncogene, respectively (14, 19–22). Our laboratory has established that IRF-1 and IRF-2 can each functionally interact with and transcriptionally activate the H4 promoter (14, 15). Furthermore, the gene for p21WAF1/CIP1, a member of the family of cyclin-dependent kinase (CDK) inhibitors, which plays a primary role in cell cycle control, is regulated in response to DNA damage by both IRF-1 and p53 (23–25). These observations suggest that the transcriptional properties of IRF-1 and IRF-2 are linked to their cell growth regulatory potential.

Cell cycle control of histone gene transcription at the onset of S phase is required for the functional coupling of histone gene expression and DNA replication (26, 27). Transcriptional control of the human histone H4 gene designated FO108 (28) has been extensively studied. The H4 gene is regulated by two multipartite proximal promoter elements (sites I and II), which together with two distal auxiliary domains (sites III and IV) modulate histone H4 promoter activity (27). Site II mediates cell cycle control of histone H4 transcription by interacting with three distinct factors, including IRF-2/HiNF-M, CDP-cut/HiNF-D, and HiNF-P (14, 29–33). The cell cycle element (CCE), 5'-CTTTCCGGTTTT-3', which is located in the distal
part of site II (34) and controls transcription at the G1/S phase transition (29), is known to interact functionally with both IRF-1 and IRF-2 (14, 15).

Recently, other IRF proteins (e.g., IRF-3 and IRF-7) have been shown to contribute to transcriptional control via IRF-binding sites. For example, the formation of distinct heterodimers between activated IRF-3 and IRF-7 may lead to differential regulation of the IFN-α and IFN-β genes (2, 35) which were initially characterized as responsive to IRF-1 and IRF-2. Both IRF-3 and IRF-7 are constitutively present in several cell types and can be activated in response to different biological stimuli, including viral infection, type I interferons, and/or DNA damage (2). These recent findings necessitate evaluation of the extent to which distinct combinations of IRF proteins may regulate histone H4 gene expression. To investigate the role of multiple IRF members in histone H4 gene transcription, we performed transfection studies with H4 promoter-luciferase reporter genes and a panel of IRF expression vectors. Our results suggest that IRF-1, IRF-2, IRF-3, and IRF-7 can all actively regulate histone H4 gene expression and that specific IRF pairs (i.e. IRF-1/IRF-3 and IRF-1/IRF-7) are strong activators.

**MATERIALS AND METHODS**

*Preparation of H4-luciferase Reporter Gene Constructs and IRF Expression Vectors—* The wild type H4 promoter/luciferase reporter gene construct wH4/Luc was derived from pFO108 wt/CAT (30, 31, 36), which contains the proximal promoter region of the H4 gene (nucleotides −240 to −38 relative to the ATG start codon; mRNA cap site at nucleotide −30) and spans sites I and II. The CAT gene was removed by PsI and HindIII cleavage and replaced by a 1.65-kilobase PsI/HindIII fragment spanning the luciferase (Luc) gene. The Luc gene was amplified from pGL3 (Promega, Madison, WI) by polymerase chain reaction amplification with two primers: forward PsI primer, 5′-gatctggagAGGGTTTCGAGCTTGCTTTTCAGCTTTCGGTTTTCAGATCCGCTTTCGGTTTTCA; reverse HindIII primer, 5′-gaacctACACGGGCATTTTCC-3′; lower case nucleotides were added to create restriction sites. The H4/Luc construct in which the IRF binding site is mutated (IRF mutH4/Luc) was prepared from pMSP16-CAT (33) and the CAT gene was excised for the Luc reporter as described above. The 4X IRF/H4-Site II/Luc plasmid was constructed by inserting an oligonucleotide cassette containing a tandemly repeated IRF consensus element spanning the distal segment of H4 site II (5′-TTTCAGCTTTCGGTTTTCA-3′) into the IRF-binding site of pGL3 (Promega) by polymerase chain reaction and cloning into pGL3 (Promega). The CCE within site II of the histone H4 gene promoter has previously been shown to interact with IRF-1 and IRF-2 (14) and is high similarity to the IRF-E and ISRE consensus elements. To determine whether more recently iden-
tified IRF family members (e.g. IRF-3 and IRF-7) are also capable of binding to the histone H4 promoter, we performed protein-DNA interaction studies with IRF proteins produced by coupled in vitro transcription and translation. The IRF-1, IRF-3, and IRF-7 proteins were analyzed by SDS-PAGE in a 10% gel for radiometric quantitation (Fig. 1, A and B). Approximately equimolar amounts of these IRF proteins were evaluated by electrophoretic mobility shift assay for binding to an oligonucleotide spanning the CCE in the distal segment of histone H4 site II (Fig. 1C). All three proteins (Fig. 1), as well as IRF-2 (Ref. 14 and data not shown), form complexes with the CCE and these complexes are competed specifically by the unlabeled wild type but not the mutant CCE oligonucleotides. The relative intensities of the signals of protein-DNA complexes suggest that IRF proteins have different affinities for the same site (IRF-1 > IRF-2 > IRF-3 = IRF-7) (Fig. 1 and data not shown). Our results indicate that in addition to IRF-2 (14), IRF-1, IRF-3, and IRF-7 are also capable of binding to the CCE in the histone H4 promoter.

Multiple IRF Proteins (IRF-1, IRF-2, IRF-3, and IRF-7) Regulate Site II-dependent H4 Promoter Activity—We have previously shown that the transcription factor IRF-2 can activate histone H4 gene expression (14) and is involved in cell cycle regulation of histone H4 gene transcription (15). To determine whether IRF-1, IRF-3, and IRF-7 function as activators or repressors of histone H4 gene expression, we performed cotransfection assays with IRF expression vectors and histone H4 gene promoter-luciferase reporter gene constructs (Figs. 2 and 3). We tested IRF-dependent activation in the context of the wild type histone H4 promoter spanning sites I and II, as well as with a mutant H4 promoter in which the IRF binding element in site II was altered by a two-nucleotide substitution that prevents IRF binding (33). The results show that IRF-3 and IRF-7 are each capable of activating transcription by 5–6-fold (Fig. 3A). For comparison, IRF-1 and IRF-2 increase transcription by ~11- and 2-fold, respectively. When the IRF-binding site was mutated, activation of the histone H4 promoter by IRF factors was completely abrogated (Fig. 3B). These results show that IRF-3 and IRF-7 can activate the histone H4 pro-
moter via the IRF recognition motif in site II.

To assess further the role of H4 site II promoter elements in mediating activation by IRF factors, we prepared two promoter constructs in which either the IRF element or the entire distal site II segment was tandemly repeated upstream of distinct minimal promoters, i.e. human histone H4 or the mouse MHC class I H2-L TATA box regions, respectively (Fig. 3, C and D). Our results indicate that IRF-1 can synergistically activate H4-related transcription in the presence of multimerized IRF elements (compare 36–60-fold activation in Fig. 3, C and D, with 11-fold activation in Fig. 3A). In contrast, IRF-2, IRF-3, and IRF-7 show approximately the same levels of activity for both the wild type histone H4 promoter and the multimerized IRF promoter constructs. Thus, it appears that IRF-1, but not other IRFs, can synergize with itself to up-regulate H4 site II-related transcription.

Dose-dependent Activation of H4 Site II-dependent Transcription by IRF Proteins—The differences in site II-dependent transcriptional activation observed for the four IRF proteins may be influenced by the relative levels of these factors. Therefore, we analyzed reporter gene activity (3X H4 distal site II/Luc) construct at different levels of each IRF factor by monitoring transcription as a function of time after transfection (Fig. 4) or concentration of expression vector (Fig. 5). The results show that IRF-related transcriptional activation by IRF-1, IRF-3, and IRF-7, but not IRF-2, is dramatically increased as these proteins accumulate at later times (e.g. 10–24 h) after transfection (Fig. 4). To relate IRF-dependent activation directly to protein levels, we performed Western blot analysis of cells transfected with different amounts of expression constructs.
and also monitored IRF activity by measuring reporter gene expression in parallel. Western blot analysis demonstrates that increasing the amount of IRF expression vector results in the expected elevation of cellular IRF proteins (Fig. 5, right panels). Reporter gene assays reveal that IRF-1-dependent activation through site II reaches saturation at a relatively modest level (0.4 μg) of IRF-1 expression vector, whereas with increasing IRF-3 and IRF-7 levels, transcriptional activity continues to increase (Fig. 5, left panels). These data are consistent with the apparent affinities of the IRF factors for site II (see Fig. 1) in that the protein with the highest affinity (i.e. IRF-1) may reach binding site saturation inside the cell at lower protein concentrations than IRF-3 and IRF-7.

Interestingly, increasing concentrations of IRF-2 result in enhancement of H4-site II driven promoter activity at low dosage, but not at higher levels (Fig. 5B). At the highest concentrations tested, IRF-2 does not display transactivation potential. These data indicate that IRF-2 is only transcriptionally active in a very narrow concentration range. Taken together, our IRF titration results indicate that these proteins have highly distinct concentration-dependent activity profiles. These distinct profiles suggest that differences in transcription observed on the wild type H4 promoter and site II related test promoters (see Fig. 3) can be accounted for by both IRF protein concentration and intrinsic transactivation potential.

IRF-1/IRF-3 and IRF-1/IRF-7 Pairs Are Strong Activators of H4 Site II-dependent Transcription—Recently it has been shown that IRF-3 and IRF-7 can function together in the regulation of the IFN-β promoter (35). To assess whether IRF-3 and IRF-7 are capable of co-regulating histone H4 gene transcription together with IRF-1 or IRF-2, we performed co-transfection assays with pairwise combinations of IRF proteins (Fig. 5).
The experiments show that co-expression of increasing amounts of IRF-2 in the presence of a fixed amount of IRF-1, IRF-3, or IRF-7 results in either a minor decrease or no effect on promoter activity (Fig. 6, A, C, and D). When IRF-2 concentrations are maintained at a constant level in the presence of increasing amounts of IRF-1, IRF-3, or IRF-7, enhancement of promoter activity is observed (Fig. 6B). These findings are consistent with limited competition of the IRF-2 protein with IRF-1, IRF-3, or IRF-7, each of which has higher activation potential at H4 site II than IRF-2. Strikingly, all pairwise combinations of IRF-1 with either IRF-3 or IRF-7 yield significantly stronger activation of reporter gene expression than any one of these three factors by itself (Fig. 6). For comparison, the combination of IRF-3 and IRF-7 (Fig. 6, C and D) results in quantitatively modest levels of reporter gene transcription. We conclude from these data (Fig. 6) that IRF-1/IRF-3 and IRF-1/IRF-7 pairs are the strongest activators of H4 site II-dependent transcription.

**INTERFERON-γ Selectively Up-regulates Histone H4 Site II-dependent Transcription**—Upon establishing that IRF-1 in combination with IRF-3 and IRF-7 is a strong enhancer of cell cycle controlled histone H4 gene transcription, we addressed the possibility that H4 gene promoter activity may be responsive to signaling mechanisms that up-regulate IRF-1. To address whether H4 site II is capable of integrating IRF-related cellular responses, we transfected cells with H4/Luc constructs and treated cells with interferon-γ (Fig. 7). When transfected mouse NIH3T3 cells were treated with mouse interferon-γ (0.5 ng/ml), we observed a strong 4–5-fold up-regulation of H4 site II-driven luciferase activity (Fig. 7, A and B) which did not occur when the IRF element within Site II was mutated (Fig. 7B). Consistent with the species specificity of interferon-γ signaling, the same concentration of human interferon-γ did not influence reporter gene activity in mouse NIH3T3 cells (Fig. 7A). We also did not observe effects of mouse interferon-γ on a promoter-less construct (pGL2) or an unrelated promoter (CDK2/Luc) (Fig. 7B). Interferon-γ did not further enhance site II-dependent transcription when cells were preloaded with IRF-1 or IRF-7 (Fig. 7C). This desensitization of interferon-γ is consistent with IRF-1 being a downstream mediator of interferon-γ effects on site II. Consistent with this concept, we find that interferon-γ significantly increases IRF-1 protein levels in NIH3T3 cells (Fig. 7D). Taken together, interactions of IRF-1 and other IRF factors with the cell cycle regulatory element in site II of the histone H4 gene may support interferon-γ-dependent cell signaling mechanisms.

**DISCUSSION**

Cell cycle control of histone H4 gene transcription involves a 3-fold enhancement of promoter activity at the G1/S phase transition and is mediated by the CCE. The results of this study show that the CCE represents a universal IRF recognition motif capable of interacting with all IRF members analyzed to date (i.e., IRF-1, -2, -3, and -7). We find that these IRF factors can each up-regulate histone H4 gene transcription and that IRF-1 is a stronger activator than IRF-2, IRF-3, or IRF-7.
More interestingly, pairwise combinations of IRF-1 with either IRF-3 or IRF-7 mediate the strongest level of activation. Thus, dimeric IRF interactions with the CCE may function to integrate signals of two distinct classes of IRF factors. The multitude of IRF factors involved in diverse immunological, genotoxic, and cell growth regulatory functions may provide a broad spectrum of gene regulatory options to control H4 gene transcription during the cell cycle.

We have previously shown that IRF-2 is identical to HiNF-M, the major DNA-binding protein that interacts with the CCE within H4 site II in HeLa S3 cells. Cell cycle analyses using mouse fibroblasts in which the genes for IRF-1 and/or IRF-2 have been ablated show that IRF-2 is important for cell cycle regulation of histone H4 gene expression (15). The deregulation of histone gene expression in these cells may be due to direct effects on the promoter or to a general effect of the IRF-2 oncoprotein on cell growth. Studies using human HeLa cervical carcinoma cells have revealed that abolishing the IRF/site II interaction by mutating the CCE has a major effect on basal level transcription (29, 33), but does not affect the timing of transcription (43). These data suggest that the role of IRF-2 in histone gene expression may be related to the growth regulatory phenotype of the cell and is possibly subject to compensatory mechanisms. The main finding in this study is that different IRF members can substitute for IRF-2 in modulating transcription, which may reflect the in vivo complexity of cell cycle regulatory mechanisms at the site II element.

IRF-2 is a weak activator of histone H4 gene transcription (14) and is also known to activate other genes, including EBNA/Qp and vascular cell adhesion molecule-1 (16, 17, 44). For some genes, IRF-2 has been shown to inhibit IRF-1 activation (4, 13), ostensibly by competing with IRF-1 for IRF-binding sites. The data presented here indicate that IRF-2 has a biphasic transcriptional activity curve in response to elevations in IRF-2 levels. Activation of the H4 gene is only observed in cells expressing low levels of IRF-2, and IRF-2 becomes transcriptionally neutral at high dosage. Thus, the dual role of IRF-2 in both transcriptional activation and inhibition of different genes may in part be attributable to cellular IRF-2 levels. The biphasic response of IRF-2 in modulating H4 gene transcription is consistent with data on IRF-2 dependent activation of the EBNA/Qp promoter in different cell types, which displays a similar dependence of promoter activity on IRF-2 concentrations (45).

In this study, we have shown that IRF-1 by itself is the strongest transactivator of histone H4-related transcription when compared with IRF-2, IRF-3, and IRF-7. IRF-1 has been shown to regulate the genes for IL-4, IL-5, IL-7 receptor, IL-12, guanylate-binding protein, EBNA/Qp, cyclin D1, ICAM 1, p53, ICE, 2–5A synthetase, p21, c-Myb, IRF-2, as well as H4 (10, 13–16, 19, 21, 22, 25, 46–48). The strength by which IRF-1 can activate transcription via the CCE suggests that the H4 gene is a physiological target of IRF-1 at least in some biological circumstances. IRF-1 dependent control of H4 gene expression would be consistent with the cell growth and/or apoptotic properties of this protein (1, 2). One novel finding of our study is that IRF-3 and IRF-7 are also capable of mediating CCE-related control of H4 gene transcription and that these proteins, when paired with IRF-1, are the strongest modulators of promoter activity. Hence, the H4 gene appears to be targeted by heterodimers comprised of IRF-1/IRF-3 and IRF-1/IRF-7.

IRF-3 and IRF-7 are phosphorylated in virus-infected cells.
Phosphorylation is required for nuclear-cytoplasmic transport of IRF-3 and IRF-7, transcriptional activation, and association of IRF-3 with p300 (35, 49–54). IRF-7 was first described to bind and repress the Qp promoter region of the Epstein-Barr virus encoded gene EBNA-1 which contains an ISRE-like element (44, 55). Recent results indicate that IRF-3 and IRF-7 can be detected in both uninfected and virus-infected cells and that IRF-7 expression is up-regulated by type I IFNs, lipopolysaccharide, and virus infection (35, 56). Our results clearly indicate that IRF-3 and IRF-7 together with IRF-1 activate the histone H4 gene promoter in the absence of viral infection.

Recent studies have indicated that IRF family members form homo- and/or heterodimers and upon binding to DNA can regulate the same gene. For example, IRF-8 forms multiple protein complexes with both IRF-1 and IRF-2 (57, 58), while IRF-3 and IRF-7 form dimers upon binding to the IFNβ promoter (52, 56, 59, 60). Similar complexes were reported for IRF-1 and/or IRF-2 (16, 38, 61, 62). Our protein/DNA interaction data, as well as results from transient co-expression experiments, suggest that IRF family members may also form dimers at the CCE to regulate histone H4 gene expression. These combined observations reinforce the idea that protein-protein complexes play central roles in the ability of IRFs to bind their cognate target genes and mediate biological functions. We conclude that the multiplicity of IRF family members apart from roles in virus-mediated signaling perform essential functions in the regulation of cell growth control in normal and tumor cells.

IFN-γ is a multifunctional cytokine with a highly cell-type dependent activity that plays an important role in immunity and cell growth control (63–67). For example, IFN-γ mediates activation of an antiviral state and regulates cell growth (64, 68). Furthermore, IFN-γ is capable of inducing cell cycle arrest and apoptosis in primary hepatocytes, but not in hepatoma cell lines (HepG2, H4IIE, and Hepa1–6) (63). Interferon-γ can stimulate cell growth depending on serum levels in malignant human T cells (68). Our data demonstrate that CCE dependent activity of the histone H4 gene promoter but not CDK2 promoter activity is selectively elevated in response to IFN-γ through IRF-1. IRF-1 dependent activation of interferon-γ responsive genes has also been observed for other genes (1–5). The biological effects of IFN-γ are mediated through a heterodimeric transmembrane receptor which is capable of stimulating the JAK-STAT pathway (69) and result in activation of IRF-1. Not withstanding the known role of interferon-γ in cell growth suppression of different cell types (69), these data suggest that IRFs, IFN-γ, and the CCE of the histone H4 gene are components of a cell signaling mechanism that may contribute to regulation of histone H4 gene transcription during the cell cycle in a cell-type or serum-dependent manner.

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