PU.1, Interferon Regulatory Factor (IRF) 2, and the Interferon Consensus Sequence-binding Protein (ICSBP/IRF8) Cooperate to Activate NF1 Transcription in Differentiating Myeloid Cells*

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NF1 (neurofibromin 1) is a Ras-GAP protein that regulates cytokine-induced proliferation of myeloid cells. In previous studies, we found that the interferon consensus sequence-binding protein (ICSBP; also referred to as interferon regulatory factor 8) activates transcription of the gene encoding NF1 (the NF1 gene) in differentiating myeloid cells. We also found that NF1 activation requires cytokine-stimulated phosphorylation of a conserved tyrosine residue in the interferon regulatory factor (IRF) domain of ICSBP/IRF8. In this study, we found that ICSBP/IRF8 cooperates with PU.1 and interferon regulatory factor 2 to activate a composite ets/IRF-cis element in the NF1 promoter. We found that PU.1 binds directly to the NF1-cis element, and DNA-bound PU.1 interacts with IRF2, recruiting IRF2 to the cis element. This interaction requires cytokine-induced phosphorylation of specific serine residues in the PU.1 PEST domain and of a conserved tyrosine residue in the IRF domain of IRF2. We found that ICSBP/IRF8 interaction with the NF1-cis element requires pre-binding of PU.1 and IRF2. The conserved IRF domain tyrosine in ICSBP/IRF8 is required for interaction with the DNA-bound PU.1-IRF2 heterodimer. NF1 deficiency in myeloid progenitor cells results in cytokine hypersensitivity and myeloproliferation. Therefore, these studies identify a target gene for the previously observed tumor-suppressor effect of PU.1. Additionally, these studies identify a tumor-suppressor function for the “oncogenic” transcription factor, IRF2.

Neurofibromin 1 (NF1) is 2818-amino acid protein with Ras-GAP activity encoded by the NF1 gene (1). In myeloid progenitor cells and differentiating phagocytes, NF1-Ras-GAP activity antagonizes granulocyte-macrophage colony-stimulating factor, stem cell factor, or macrophage colony-stimulating factor-induced Ras activation (2–4). Therefore, NF1-deficient cells exhibit cytokine hypersensitivity (i.e. increased proliferation to low cytokine doses). Congenital NF1 deficiency is associated with neurofibromatosis (5) and juvenile myelomonocytic leukemia (6). Acquired NF1 deficiency has been documented in myeloid cells from human subjects with acute myeloid leukemia (AML)2 and myelodysplastic syndromes (MDS) (7). Therefore, although NF1 expression is not restricted to hematopoietic cells, NF1 deficiency is implicated in the pathogenesis of malignant myeloid disorders. Consistent with this, NF1 deficiency induces a myeloproliferative disorder in murine models (8, 9).

Previously, we found that NF1 transcription and NF1 expression increase during cytokine-induced differentiation of myeloid leukemia cell lines or murine myeloid progenitor cells (4). We also found that cytokine-induced NF1 transcription requires the interferon consensus sequence-binding protein (ICSBP or IRF8) (4). ICSBP/IRF8 is expressed exclusively in myeloid and B-cells (10), and acquired ICSBP/IRF8 deficiency is found in bone marrow cells from subjects with chronic myeloid leukemia, AML, and MDS (11, 12). Interestingly, ICSBP/IRF8 deficiency induces myeloproliferation in mice, and myeloid cells from these mice are hypersensitive to the same cytokines as NF1-deficient cells (4, 13, 14). Consistent with a role in NF1 transcription, proliferative abnormalities in ICSBP/IRF8-deficient myeloid progenitor cells can be rescued by expression of the NF1GAP-related domain (4).

In previous studies, we also found that activation of NF1 transcription requires cytokine-induced phosphorylation of a specific ICSBP/IRF8 tyrosine residue (Tyr-95) (15). This residue is in the conserved IRF domain that is thought to be involved in DNA-binding or protein/protein interactions of IRF proteins. ICSBP/IRF8 is a substrate for SHP1 and SHP2 protein-tyrosine phosphatases in undifferentiated myeloid cells (15, 16). However, a constitutively activated SHP2 mutant, described in human subjects with MDS, AML, and juvenile myelomonocytic leukemia, dephosphorylates ICSBP/IRF8 in differentiated and undifferentiated myeloid cells (15, 17). Such activated SHP2 mutants also induce cytokine hypersensitivity in myeloid progenitors (15, 18).

IRF proteins regulate target gene transcription by interacting with several different DNA-binding site consensus sequences. ICSBP/IRF8 represses cis elements with PRDI consensus sequences (5′-TCACTT-3′) by interacting directly with DNA.

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2 The abbreviations used are: AML, acute myeloid leukemia; ICSBP, interferon consensus sequence-binding protein; IRF2, interferon regulatory factor 2; GST, glutathione S-transferase; MDS, myelodysplastic syndromes; IFN, interferon; EMSA, electrophoretic mobility shift assay; CREB, cAMP-response element-binding protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DAPA, DNA affinity purification assay; shRNA, short hairpin RNA; ds, double stranded; ERK, extracellular signal-regulated kinase; CAT, chloramphenicol acetyltransferase.
In contrast, tyrosine-phosphorylated ICSBP/IRF8 can activate PRDI-cis elements by interaction with DNA-bound IFI1 (19). ICSBP/IRF8 also binds to interferon-stimulated response elements (5′-GAAANNGAAA-3′). Interferon-stimulated response elements can be repressed by an interaction between ICSBP/IRF8, the ets protein Tel, and histone deacetylase 3 (20).

ICSBP/IRF8 activates gene transcription by interacting with composite ets/IRF-cis elements (also referred to as EICE sequences). Such ets/IRF sequences have a loose consensus (5′-GGAA(A/G)TGNNNA-3′) and are found in a number of genes expressed in mature phagocytes and involved in the immune response. Examples include genes encoding the respiratory burst oxidase proteins gp91PHOX and p67PHOX and the gene encoding the Toll-like receptor 4 (the CYBB, NCF2, and TLR4 genes, respectively) (11, 21, 22). DNA-bound PU.1 interacts with IRF1, and this DNA-bound heterodimer recruits ICSBP/IRF8 and the CREB-binding protein (11, 21, 22). These interactions require post-translational modification of the proteins in differentiating myeloid cells. Specifically, interaction of ICSBP/IRF8 with the DNA-bound PU.1-IRF1 heterodimer requires phosphorylation of PU.1 Ser-148 and ICSBP/IRF8 Tyr-95 (16, 21).

The ICSBP/IRF8-binding cis element in the proximal NF1 promoter has homology to composite ets/IRF consensus sequences found in myeloid-specific genes. This suggests possible involvement of PU.1 in NF1 transcriptional regulation and perhaps another IRF protein. The goal of these investigations is to determine the mechanism of cytokine-induced NF1 transcription in differentiating myeloid cells. This will be approached by identifying the components of the NF1 transcriptional activation complex.

Although composite ets/IRF consensus sequences have been identified in a number of genes involved in the inflammatory response, no such cis elements have previously been identified in target genes regulating proliferation. Identification of homologous cis elements that interact with common trans-factors in genes that regulate both differentiation and proliferation would suggest a common mechanism of cytokine activation of different types of genes. This could have implications for understanding the inter-relationship between differentiation-progression and proliferation-arrest during myelopoiesis.

**MATERIALS AND METHODS**

**Plasmids and PCR Mutagenesis**

**Protein Expression Vectors**—The ICSBP/IRF8 cDNA was obtained from Dr. Ben Zion-Levi (Technion, Haifa, Israel), and the full-length cDNA was generated by PCR and subcloned into the mammalian expression vector pcDNAAmp, as described (21). The cDNA for IRF2 in the pcDNAAmp vector was obtained from Dr. Gary S. Stein (University of Massachusetts Medical School, Worcester, MA). IRF2 with mutation of a conserved tyrosine residue in the IRF domain (Tyr-109) to phenylalanine was generated by PCR using the Clontech “QuikChange” protocol, as described (16). Mutant clones were sequenced on both strands to verify that only intended mutations had been introduced. Wild type PU.1 and PU.1 mutants with serine 41 and 45, 148, or 132 and 133 changed to alanine were obtained from Dr. Michael L. Atchison (University of Pennsylvania School of Veterinary Medicine, Philadelphia) and subcloned into the pSRa mammalian expression vector. These cDNAs were also subcloned into the pGEX1 vector (Amersham Biosciences) for expression in *Escherichia coli* as glutathione S-transferase (GST) fusion proteins.

**Reporter Constructs**—An artificial promoter construct with four copies of the ICSBP/IRF8-binding cis element from the NF1 promoter (bp −320 to −336) linked to a minimal promoter and a CAT reporter (the p-TATACAT vector) was previously described (15). This construct is referred to as nftTATACAT.

**Oligonucleotides**

Oligonucleotides were custom-synthesized by MWG Biotec (Piedmont, NC). Double-stranded oligonucleotides used in EMSA and DNA affinity co-immunoprecipitation assays represent the −320 to −336 bp NF1 promoter sequence (NF1−320 to −336; 5′-ggatccactctcggtgcc-3′). The underlined sequence has homology to composite ets/IRF-cis elements from other ICSBP target genes. A double-stranded oligonucleotide with mutation of the ICSBP/IRF8-binding site (previously described (4)) was also used in these assays (mutNF1−320 to −336; 5′-ggatccccacacgggtgcc-3′). For in vitro binding competition assays, an oligonucleotide with the composite ets/IRF sequence for the CYBB gene was used (5′-gtttcactctctggc-3′) (21). Subcloning these oligonucleotides into a plasmid vector to generate probes has been described (4).

**Myeloid Cell Line Culture**

The human myelomonocytic cell line U937 (24) was obtained from Andrew Kraft (Hollings Cancer Center, Medical University of South Carolina, Charleston, SC). Cells were maintained and differentiated as described (21). For differentiation experiments, U937 cells were treated for 24 or 48 h with 500 units/ml human recombinant IFNγ (Roche Applied Science) (21).

**Transfections and Reporter Gene Assays**

U937 cells were cultured and transfected as described previously (4, 15, 21, 22). Cells (32 × 10⁶ per sample) were transfected with a vector to express the following: wild type ICSBP/IRF8, Y95F ICSBP/IRF8, or empty vector control; wild type IRF2, Y109F IRF2, or empty vector control; wild type PU.1, S148A PU.1, S132A/S133A PU.1, or empty vector control; the minimal promoter/reporter vector pTATACAT with four copies of the −320 to −336 bp NF1 sequence (nftTATACAT) or empty vector control (pTATACAT); and p-CMVβgal (to control for transfection efficiency). Transfectants were harvested 48 h after transfection, with or without incubation with recombinant human IFNγ (500 units/ml). Lysates were analyzed for CAT and β-galactosidase activity, as described (21).

**Isolation of Nuclear Proteins and Electrophoretic Mobility Shift Assays**

Nuclear extract proteins were isolated from U937 cells by the method of Dignam et al. (25) (with the addition of protease inhibitors but not phosphatase inhibitors, as described). In
some experiments, U937 cells were differentiated with 500 units/ml of IFN-γ before nuclear protein isolation. Oligonucleotides probes were prepared, and EMSA and antibody supershift assays were performed, as described (21). Antibodies to phosphotyrosine, ICSBP/IRF8, IRF1, IRF2, PU.1, and irrelevant, control GST antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

**Immunoprecipitation and Western Blots**

*Western Blots of U937 Lysates Proteins—*U937 cells were lysed by boiling in 2× SDS sample buffer. Lysate proteins (30 μg) were separated by SDS-PAGE (12% acrylamide) and transferred to nitrocellulose, according to standard techniques. Western blots were serially probed with antibodies to NF1, ICSBP/IRF8, IRF2, PU.1, and GAPDH (to control for loading). In other studies, nuclear proteins were isolated from U937 cells (with or without IFN-γ treatment). 30 μg of protein were separated by SDS-PAGE (20% acrylamide gel) and transferred to nitrocellulose. Western blots were serially probed with antibodies to PU.1, IRF2, pERK2 (as a control for IFN-γ treatment), total ERK2 (as a loading control), or GAPDH (as a loading control).

*Immunoprecipitation and Western Blots—*Nuclear proteins were isolated from U937 cells with or without IFN-γ treatment and immunoprecipitated under denaturing conditions with antibody to PU.1, IRF2, phosphotyrosine (Tyr(P)), or irrelevant control antibody (anti-GST), as described previously (14, 15). Precipitated proteins were collected with staphylococcus protein A-Sepharose, separated by SDS-PAGE, and transferred to nitrocellulose, as above. Western blots were serially probed with a phosphotyrosine antibody (clone 4G10, Upstate) and antibodies to PU.1 or irrelevant GST antibody. Immunoprecipitates were collected with staphylococcus protein A-Sepharose beads and separated by SDS-PAGE (20% acrylamide). Phosphorylated PU.1 protein was identified by autoradiography of the fixed and dried gel.

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*In Vitro Protein Translation and GST Co-affinity Purification Assay—*In vitro transcribed IRF2, Y109F IRF2, ICSBP/IRF8, Y95F ICSBP/IRF8, PU.1, S148A PU.1, or S132A/S133A PU.1 mRNAs were generated from linearized template DNA using the riboprobe system, according to manufacturer’s instructions (Promega). In vitro translated proteins were generated in rabbit reticulocyte lysate, according to the manufacturer’s instructions (Promega). Control (unprogrammed) lysates were generated in similar reactions in the absence of input RNA, and proteins were radiolabeled by including [35S]methionine in the translation reaction. JM109 E. coli transformed with PU.1, IRF2, or ICSBP/IRF8 in the pGEX vector (or empty control vector) were grown to log phase, supplemented to 0.1 mM isopropyl-β-d-galactopyranoside, and incubated for 3 h at 37 °C with shaking. The cells were harvested and resuspended in HN buffer (20 mM HEPES (pH 7.4), 0.1 mM NaCl, 2 mM MgCl2, 0.1 mM EDTA, 0.5% Nonidet P-40, 0.1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, 5 mM NaF) and sonicated on ice (22). Debris was removed by centrifugation, and the lysate was incubated with glutathione-agarose beads (Sigma) and washed extensively. The beads were preincubated with control rabbit reticulocyte lysate to induce serine/threonine phosphorylation of PU.1/GST and tyrosine phosphorylation of ICSBP/GST or IRF2/GST as described (16, 26). GST proteins were then incubated with [35S]methionine-labeled *in vitro* translated protein. Proteins were eluted with SDS-PAGE sample buffer, separated on 15% SDS-PAGE, and identified by autoradiography of the fixed and dried gel.

**RESULTS**

*The Positive NF1-cis Element Binds a Multiprotein Complex—*IFN-γ treatment of U937 cells increases NF1 promoter activity and ICSBP/IRF8 binding to a positive cis element in the NF1 promoter (4, 15). To identify other proteins that interact with this cis element, we used *in vitro* DNA binding assays. EMSAs were performed with a radiolabeled, double-stranded oligonucleotide representing the NF1-cis element and nuclear proteins from U937 cells. We found that IFN-γ differentiation of U937 cells increases *in vitro* protein binding to this probe, consistent with our previous results (Fig. 1A) (4, 15).

We noted that the NF1-cis element has homology with the ets/IRF consensus sequences from the CYBB or NCF2 promoters. Therefore, we used double-stranded oligonucleotide competitors representing these cis element to investigate binding specificity of the NF1-protein complex. These competitors were compared with homologous oligonucleotide (ds NF1) or an oligonucleotide with mutation which abolishes protein binding to the NF1-cis element (ds mutNF1) (described (4)). In EMSA with the NF1 probe and nuclear proteins from IFN-γ-differentiated U937 cells, we found that the CYBB and NCF2 oligonucleotides compete for protein complex binding (Fig. 1B). This result suggests that the NF1-cis element interacts with PU.1 and one or more IRF proteins.
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Therefore, we performed EMSA with the NF1 probe, nuclear proteins from IFNγ-treated U937 cells, and antibodies to various transcription factors (Fig. 1C). In these studies, we found that the shifted complex is cross-immunoreactive with PU.1, ICSBP/IRF8, and IRF2 but not IRF1. Because the NF1-cis element appears to bind a multiprotein complex, we tested the ability of various combinations of PU.1, IRF2, and ICSBP/IRF8 antibodies to disrupt the complex (Fig. 1C). We found that the complex is completely disrupted by combining all three antibodies.

We also used DAPA to determine the impact of differentiation on assembly of the multiprotein complex on the NF1-cis element. To determine specificity of binding, wild type NF1-cis element probe (ds NF1) was compared with the binding-mutant probe (ds mutNF1). These oligonucleotides were biotin-labeled and incubated with U937 nuclear proteins. Probes were precipitated with anti-biotin antibody, and co-precipitating proteins were separated by SDS-PAGE and identified by Western blot (Fig. 1D).

In this assay, we also find that IFNγ treatment increases interaction of PU.1, IRF2, and ICSBP/IRF8 with the NF1-cis element probe. In contrast, we found previously that PU.1 and IRF1 bind the CYBB- and NCF2-cis elements in undifferentiated myeloid cells. For those cis elements, differentiation increases ICSBP/IRF8 interaction with these proteins but does not increase DNA binding of PU.1 and IRF1. Our current results suggest differences in the activation of various ets/IRF-cis elements, even in the same lineage.

Identification of Protein/Protein/DNA Interactions Required for Assembly of the NF1-cis Element-binding Complex—Previous studies suggest that PU.1 binding to ets/IRF consensus sequences is required to provide an anchor for IRF proteins. Therefore, we investigated the role of PU.1 in mediating IRF2 and ICSBP/IRF8 binding to the NF1-cis element. For these studies, we used in vitro translated, 35S-labeled PU.1, IRF2, and ICSBP/IRF8 (Fig. 2A). Because of differences in size, the three proteins can be detected simultaneously by SDS-PAGE. These in vitro translated proteins were tested for binding to biotin-labeled ds NF1 or ds mutNF1 probes in DNA-affinity purification assays (DAPA), as above. In these experiments the total amount of reticulocyte lysate in the binding assays was kept constant by inclusion of control (no RNA) lysate.

In initial assays, we tested each of these proteins individually for binding to the ds NF1 probe (Fig. 2B). We found that only
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PU.1 is able to interact independently with this probe, and none of the proteins interact with the ds mutNF1 probe. Previous studies indicate that phosphorylation of serine residues in the PU.1 PEST domain is important for PU.1 protein/protein/DNA interactions. For example, PU.1 serine 148 is necessary for recruitment of ICSBP/IRF8 to the CYBB promoter (21). Similarly, serines 132 and 133 in PU.1 are essential for PU.1 activation of macrophase-specific genes (27). Because these residues are phosphorylated in reticulocyte lysate (28, 29), we used DAPA to investigate the role of PU.1 phosphorylation in the assembly of the NF1-binding protein complex. We found that in vitro translated wild type PU.1 and PU.1 with mutation of serine 148 or 132/133 to alanine (S148A PU.1 and S132A/S133A PU.1, respectively) have similar binding to the NF1-cis element probe (Fig. 2C). This result is important for interpretation of transfection experiments as shown in the sections below.

We next investigated whether PU.1 recruits either IRF protein to the NF1-cis element. Biotin-labeled probe was incubated with PU.1 and either ICSBP/IRF8 or IRF2 (Fig. 2D). We found that PU.1 recruits IRF2 to the NF1-cis element but not ICSBP/IRF8. Therefore, we tested the effect of PU.1 PEST domain residues on interaction with IRF2 by DAPA (Fig. 2D). In these studies, DNA-bound wild type and S148A PU.1 interact with IRF2 equivalently. In contrast, IRF2 does not interact with S132A/S133A PU.1 bound to the ds NF1 probe.

We previously identified a conserved tyrosine residue in the ICSBP/IRF8 IRF domain that is necessary for interaction with a PU.1-IRF1 heterodimer bound to the CYBB- or NCF2-cis element. Because IRF domain tyrosine residues are phosphorylated in reticulocyte lysate (16), we used DAPA to determine the role of the IRF2 IRF domain tyrosine (Tyr-109) in interaction with PU.1. We incubated PU.1 and wild type or Y109F IRF2 (tyrosine 109 mutated to phenylalanine) with the biotin-labeled ds NF1 or ds mutNF1 probe (Fig. 2D). We found that Y109F IRF2 interacts less efficiently with DNA-bound PU.1 than wild type IRF2.

We next investigated whether ICSBP/IRF8 can interact with the DNA-bound PU.1-IRF2 heterodimer. We found that in vitro translated ICSBP/IRF8 interacts with the ds NF1 probe (but not the ds mutNF1 probe) in binding assays with PU.1 and IRF2 (Fig. 2E). Because the conserved IRF domain tyrosine in ICSBP/IRF8 (Tyr-95) is required for activation of the NF1-cis element, we performed binding assays to determine the role of
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Interaction of PU.1, IRF2, and ICSBP/IRF8 requires specific post-translational modification of each of the three proteins. A, S132A/S133A PU.1 has lower affinity for IRF2 than does wild type. IRF2 was expressed as a fusion protein with glutathione S-transferase in E. coli (IRF2/GST). This fusion protein and control GST were used in affinity purification assays with in vitro translated (IVT) S132A/S133A PU.1, or wild type PU.1 (GST pulldown assay). In vitro translated S132A/S133A PU.1 has less affinity for IRF2/GST than does in vitro translated wild type PU.1. Neither of these in vitro translated proteins co-purifies with control GST. B, S148A PU.1 has equivalent affinity for IRF2 than does wild type. IRF2 was expressed as a fusion protein with glutathione S-transferase in E. coli. IRF2/GST and control GST were used in affinity purification assays with in vitro translated, 35S-labeled S148A PU.1 or wild type PU.1 (GST pulldown assay). The affinities of S148A PU.1 and wild type PU.1 for IRF2/GST are equivalent. Neither of these in vitro translated proteins co-purifies with control GST. C, Y109F IRF2 has lower affinity for PU.1 than does wild type. PU.1 was expressed as a fusion protein with glutathione S-transferase in E. coli. IRF2/GST and control GST were used in affinity purification assays with in vitro translated, 35S-labeled Y109F IRF2 or wild type IRF2 (GST pulldown assay). In vitro translated Y109F IRF2 has less affinity for PU.1/GST than does in vitro translated wild type IRF2. Neither of these in vitro translated proteins co-purifies with control GST. D, Y95F ICSBP/IRF8 has lower affinity for IRF2 than does wild type. Y95F ICSBP/IRF8 was expressed as a GST fusion protein, and phosphorylated Y95F ICSBP/IRF8 was used in affinity purification assays with in vitro translated, 35S-labeled Y95F ICSBP/IRF8 or wild type ICSBP/IRF8 (GST pulldown assay). In vitro translated Y95F ICSBP/IRF8 has less affinity for IRF2/GST than in vitro translated wild type ICSBP/IRF8. Neither of these in vitro translated proteins co-purifies with control GST.

This residue in interaction with DNA-bound PU.1 + IRF2 (Fig. 2E). We found that Y95F ICSBP/IRF8 interacts less efficiently with the dsNf1 probe + PU.1 + IRF2.

Based on these results, it is possible that ICSBP/IRF8 interacts directly with IRF2 and that IRF2 interacts with DNA-bound PU.1. However, it is also possible that DNA-bound PU.1 and IRF2 form a binding site that involves interaction of ICSBP/IRF8 with both proteins. Otherwise, ICSBP/IRF8 interaction may depend on conformational changes of either PU.1 or IRF2, which occur when these two proteins interact with the DNA-binding site. Therefore, we tested the impact of mutating PU.1 serine 148 on interaction of ICSBP/IRF8 with PU.1 + IRF2 bound to the Nf1-cis element. We found that dsNf1-bound S148A PU.1 + IRF2 does not recruit ICSBP/IRF8 to the binding site as efficiently as wild type PU.1 + IRF2 (Fig. 2E).

These studies identify the order of binding of the activation complex proteins to the Nf1-cis element. We also investigated interaction between these proteins using an in vitro assay system that does not require DNA binding of component proteins. For these studies, we expressed IRF2 in E. coli as a fusion protein with glutathione S-transferase. The IRF2/GST fusion protein was phosphorylated by incubation with reticulocyte lysate, as described previously (16). We investigated interaction with in vitro translated wild type versus S132A/S133A PU.1 versus S148A PU.1 by “pulldown” assay. Previously, we found that mutation of Ser-148 impairs the ability of PU.1 to interact with GST than does Y95F ICSBP/IRF8 (Fig. 3D).

None of these in vitro translated proteins co-precipitate with control GST. Phosphorylation of GST fusion proteins and in vitro translated proteins is discussed under “Materials and Methods.”

PU.1, IRF, and ICSBP/IRF8 Cooperate to Activate Nf1 Transcription—We next investigated the functional significance of PU.1 and IRF2 for Nf1 transcription. In previous studies, we determined that overexpressed ICSBP/IRF8 activates transcription from an artificial promoter construct with multiple copies of the Nf1-cis element in U937 transfectants (15). For this study, we used the same reporter system to determine the impact of overexpressed PU.1 and IRF2 on this Nf1-cis element. Expression of IRF2 is ubiquitous, and PU.1 and ICSBP/IRF8 are expressed in all myeloid cell lines. Therefore, overexpressed ICSBP/IRF8 may have activated the Nf1-cis element in our previous studies by interacting with endogenous PU.1 and IRF2. In this case, overexpression of various combinations PU.1, IRF2, and ICSBP/IRF8 might be expected to result in more reporter gene expression than overexpression of any protein individually.

In initial experiments, U937 cells were transfected with a reporter construct containing a minimal promoter with or without four copies of the Nf1-cis element (NF1TATACAT and pTATACAT control, respectively). The cells were co-transfected with mammalian expression vectors to overexpress ICSBP/IRF8 in such assays (16). In this study, less in vitro translated S132A/S133A PU.1 co-purifies with IRF2/GST in comparison with the wild type PU.1 (Fig. 3A). In contrast, S148A PU.1 and wild type PU.1 co-purify equivalently with IRF2/GST (Fig. 3B).

We also determined the role of IRF2 IRF domain phosphorylation for interaction with non-DNA-bound PU.1. For these studies, PU.1 was expressed in E. coli as a GST fusion protein and phosphorylated by incubation with reticulocyte lysate, as described (29). Wild type and Y109F IRF2 were translated in vitro in reticulocyte lysate. We found that wild type IRF2 co-precipitates with PU.1/GST more efficiently than Y109F IRF2 (Fig. 3C).

We also performed similar experiments to pursue the hypothesis that ICSBP/IRF8 tyrosine phosphorylation is necessary for interaction with IRF2. For these studies, IRF2 was expressed as a GST fusion protein, as described above, and incubated with in vitro translated wild type or Y95F ICSBP/IRF8. We found that the wild type protein co-precipitates more efficiently with IRF2/
ICSBP/IRF8, PU.1, or IRF2 or control expression vector (Fig. 4A). Because of the impact of differentiation on the NF1-cis element, these transfectants were analyzed with and without IFNγ treatment (4, 15).

We found that ICSBP/IRF8 overexpression activates the NF1-cis element-containing reporter construct, consistent with our previous results (15). Similarly, we found that overexpression of IRF2 also significantly increases reporter activity of the NF1-cis element-containing construct in both untreated and IFNγ-differentiated transfectants ($p < 0.0001, n = 5$). PU.1 overexpression induces a smaller but statistically significant increase in reporter expression from the NF1-cis element activity in undifferentiated transfectants ($p \leq 0.04, n = 12$) and a larger increase in differentiated transfectants ($p < 0.0001, n = 12$).

Based on these results, we tested the effect of coexpressing combinations of these proteins (Fig. 4A). We found that coexpressing PU.1 with either IRF2 or ICSBP/IRF8 results in a statistically significant increase in activity of the NF1-cis element in comparison with overexpressing either IRF protein alone ($p \leq 0.002, n = 4$ for all combinations, with and without IFNγ). We also tested the effect of coexpressing all three proteins on NF1-cis element activity. We were interested in determining whether the effect of overexpressing PU.1 and both IRF proteins was different from overexpressing PU.1 and either IRF protein alone. To determine this, the total amount of IRF expression vector was kept constant when comparing PU.1 + IRF2 versus PU.1 + ICSBP + IRF2 versus PU.1 + IRF2 + ICSBP.

We found that reporter expression from the NF1-cis element-containing construct is significantly greater in transfectants with PU.1 + ICSBP + IRF2 in comparison with PU.1 + ICSBP or PU.1 + IRF2 ($p \leq 0.002, n = 7$; with and without IFNγ) (Fig. 4A). These results suggest that IRF2 and ICSBP are not functionally redundant for their impact on NF1 transcription. In control experiments, none of these proteins significantly influence reporter expression from the empty pTATACAT vector, with or without IFNγ treatment of the transfectants.

Because these studies involve expressing multiple proteins from multiple vectors, we performed a control experiment to verify that these proteins could be simultaneously overexpressed. U937 cells were co-transfected with vectors to overexpress PU.1, IRF2, and ICSBP/IRF8 or with an equivalent amount of control vector. Transfectants were incubated with IFNγ, as in the reporter gene assays above. Lysate proteins were separated by SDS-PAGE and analyzed for protein expression by Western blot (Fig. 4B). We found that these proteins are indeed co-overexpressed in U937 cells under these conditions. We also find that this co-overexpression increases expression of endogenous NF1, consistent with the reporter assays.

**PLU.1 Is Essential for Activation of the NF1-cis Element in U937 Myeloid Cells**—We hypothesize that PU.1 binding to the NF1-cis element is essential for binding of IRF2, which is essential for binding of ICSBP/IRF8. If this hypothesis is correct, decreasing endogenous PU.1 in U937 cells should decrease activity of the NF1-cis element. To investigate this hypothesis, U937 cells were co-transfected with a vector to express a PLU.1-specific short hairpin RNA (shRNA) or scrambled control shRNA. We found that expression of a PU.1-specific shRNA induces a small but significant decrease in reporter expression from the NF1-cis element in U937 transfectants with and without IFNγ differentiation ($p < 0.002, n = 3$) (Fig. 5A).

Therefore, we next determined the impact of co-overexpressing PU.1-specific shRNA with IRF2, ICSBP/IRF8, or IRF2 + ICSBP/IRF8. This experiment would test our hypothesis that activation of the NF1-cis element by overexpressed IRF2 or ICSBP/IRF8 requires the presence of either endogenous or overexpressed PU.1. We found that expression of PU.1-specific shRNA (but not control scrambled shRNA) blocks activation of the nTATACAT reporter construct by overexpressed IRF2, ICSBP/IRF8, or IRF2 + ICSBP/IRF8 (Fig. 5A). Indeed, we found that activity of the NF1-cis element containing construct is not significantly influenced by overexpression of these IRF proteins in the presence of PU.1 shRNA expression ($p = 0.61, F = 0.54, n = 3$). Expression of PU.1-specific shRNA does not impact reporter expression from pTATACAT control vector.
ICSBP/IRF8, IRF2, and PU.1 Activate Nf1 Expression

Based on these results, we also investigated the effect of PU.1 knockdown on expression of endogenous Nf1. For these experiments, U937 transfectants with PU.1-specific or scrambled control shRNA were IFNγ-treated, and cell lysates were analyzed by Western blot (Fig. 5B). We found that decreased PU.1 expression in U937 cells is associated with decreased Nf1 expression. This is consistent with the reporter gene assays.

Mutation of PU.1 PEST Domain Serine Residues Prevents Functional Interaction with IRF2 and ICSBP—We found that mutation of serine residues 132 and 133 in the PU.1 PEST domain impairs the ability of PU.1 to recruit IRF2 to the Nf1 cis-element in vitro. Based on these results, we tested the impact of S132A/S133A PU.1 on activation of Nf1 transcription. The reporter gene assays in this section were performed simultaneously with the experiments in Fig. 4A, but they are presented in separate graphs for convenience of interpretation. U937 cells were co-transfected with the Nf1 cis-element-containing reporter construct (or control pTATACAT) and a vector to express wild type or S132A/S133A PU.1 expression vector (Fig. 6A). We found S132A/S133A PU.1 induces significantly less expression from the Nf1 cis-element in comparison with wild type PU.1, with and without IFNγ-differentiation (p = 0.0002, n = 6). Indeed, reporter activity from the Nf1 cis-element containing construct is less in transfectants overexpressing S132A/S133A PU.1 + IRF2 than in transfectants overexpressing only IRF2 (p = 0.001, n = 4 for undifferentiated and IFNγ-treated transfectants). These results support the hypothesis that S132A/S133A PU.1 prevents binding of the Nf1 cis-element, therefore preventing interaction with overexpressed IRF2.

Our DNA binding assays indicate that a DNA-bound PU.1-IRF2 heterodimer is essential for interaction of ICSBP/IRF8 with the Nf1 cis-element. This suggests activation of the Nf1 cis-element by overexpressed PU.1 and ICSBP/IRF8 requires endogenous IRF2. Because IRF2 does not interact with DNA-bound S132A/S133A PU.1, one would anticipate that overexpressed S132A/S133A PU.1 + ICSBP will also not activate the Nf1 cis-element. Consistent with this, we found that S132A/S133A PU.1 + ICSBP induces significantly less reporter expression from the Nf1TATACAT construct than PU.1 + ICSBP in undifferentiated and IFNγ-treated transfectants (p = 0.0001, n = 4).

We also tested the impact of mutating PU.1 serine residues 132 and 133 on activation of the Nf1 cis-element by overexpressed IRF2 + ICSBP (Fig. 6A). We found significantly less reporter expression from Nf1TATACAT in transfectants with S132A/S133A PU.1 + IRF2 + ICSBP than in transfectants with wild type PU.1 + these two IRF proteins. This was true in transfectants both with and without IFNγ-differentiation (p =
Indeed, activity of the \(\text{NF1}\)-cis element is not significantly different in transfectants overexpressing S132A/S133A PU.1 + IRF2 + ICSBP than in transfectants with empty control expression vector \((p \cong 0.7, n = 4)\).

Our \textit{in vitro} DNA binding assays indicate that mutation of serine 148 in the PU.1 PEST domain impairs the ability of the DNA-bound PU.1-IRF2 heterodimer to recruit ICSBP/IRF8. Therefore, we investigated the impact of overexpressing S148A PU.1 on activation of the \(\text{NF1}\)-cis element. In initial experiments, we found that S148A PU.1 is significantly less efficient at activating this construct than wild type PU.1 in transfectants, with and without IFN\(\gamma\) \((p \cong 0.01, n = 8)\). These results suggest that activation of this cis element by overexpressed PU.1 involves interaction with both endogenous IRF2 and ICSBP.

Therefore, we investigated the impact of S148A PU.1 on IRF2 activation of the \(\text{NF1}\)-cis element (Fig. 6B). We found that S148A PU.1 + IRF2 is significantly less efficient than wild type PU.1 + IRF2 in activation of reporter expression from the \(\text{NF1}\)-cis element in untreated and IFN\(\gamma\)-differentiated U937 transfectants \((p \cong 0.04, n = 4)\). These results suggest that this PU.1 mutation impairs the ability of the PU.1-IRF2 heterodimer to interact with endogenous ICSBP/IRF8.

We also tested the ability of S148A PU.1 to induce \(\text{NF1}\)-cis element activation in cooperation with both overexpressed IRF2 and ICSBP/IRF8. We found that nfITATACAT expression in transfec-
tants with S148A PU.1 + the two IRF proteins is less than half that in

\[\text{ICSBP/IRF8, IRF2, and PU.1 Activate Nf1 Expression}\]

\[\text{FIGURE 6. Specific PU.1 serine residues are required for cooperation with IRF2 and ICSBP and activation of the \(\text{NF1}\)-cis element. A, overexpressed S132/133A PU.1 does not cooperate with IRF2, ICSBP/IRF8, or IRF2 + ICSBP/IRF8 to activate the \(\text{NF1}\)-cis element. U937 cells were co-transfected with an artificial promoter construct with multiple copies of the \(\text{NF1}\)-cis element (nfITATACAT) or empty vector control (pTATACAT) and various combinations of vectors to overexpress S132A/S133A PU.1 or wild type PU.1 and IRF2 and ICSBP/IRF8. Reporter gene assays were performed with or without IFN\(\gamma\) treatment of the transfectants. S132A/S33A PU.1 is less efficient at activation of the nfITATACAT construct than wild type PU.1 alone, or with IRF2, ICSBP/IRF8, and IRF2 + ICSBP/IRF8, with and without IFN\(\gamma\) treatment. None of these overexpressed proteins significantly influenced reporter expression from control pTATACAT. B, overexpressed S148A PU.1 does not cooperate with IRF2, ICSBP/IRF8, or IRF2 + ICSBP/IRF8 to activate the \(\text{NF1}\)-cis element. U937 cells were co-transfected with an artificial promoter construct with multiple copies of the \(\text{NF1}\)-cis element (nfITATACAT) or empty vector control (pTATACAT) and various combinations of vectors to overexpress S148A PU.1 or wild type PU.1 and IRF2 and ICSBP/IRF8. Reporter gene assays were performed with or without IFN\(\gamma\) treatment. S148A PU.1 is less efficient at activation of the nfITATACAT construct than wild type PU.1 alone or with IRF2, ICSBP/IRF8, and IRF2 + ICSBP/IRF8, with and without IFN\(\gamma\) treatment. None of these overexpressed proteins significantly influenced reporter expression from control pTATACAT.}\]
transfectants with wild type PU.1 + IRF2 + ICSBP/IRF8 (p = 0.001, n = 4). This effect is even greater in IFN-γ-treated transfectants (p < 0.00002, n = 6).

We found that there is no significant difference in activation of nf1TATACAT reporter activity by S148A PU.1 in comparison with S132A/S133A PU.1 (p = 0.61, n = 8 and p = 0.98, n = 8, respectively). Similarly, we found that there is no significant difference in NF1-cis element activation in transfectants with S148A PU.1 + ICSBP/IRF8 + IRF2 versus S132A/S133A PU.1 + ICSBP/IRF8 + IRF2, with and without IFNγ treatment (p = 0.1, n = 8 and p = 0.99, n = 8, respectively). These results suggest that impairing ICSBP/IRF8 interaction with the NF1-cis element has the same effect, whether it is because of impairing interaction between PU.1 and IRF2 (by mutating Ser-132 and Ser-133) or by impairing interaction of ICSBP/IRF8 with the PU.1-IRF2 heterodimer.

Expression from the empty reporter vector pTATACAT is not significantly altered by expression of any of these proteins. Previous studies and our own preliminary data indicate that expression of wild type PU.1 and various serine mutants is equivalent in such transfection experiments (not shown in this study) (26).

Mutation of a Conserved IRF Domain Tyrosine Prevents IRF2 from Activating NF1 Transcription—We found that the conserved IRF domain tyrosine in IRF2 increases interaction with DNA-bound PU.1. Therefore, we investigated the role of this residue (Tyr-109 IRF2) in activation of the NF1-cis element. We performed U937 transfection experiments with the NF1-cis element-containing minimal promoter/reporter construct or pTATACAT control vector and vectors to overexpress various combinations of wild type or Y109F IRF2, ICSBP, and PU.1 (Fig. 7A). The reporter gene assays in this section were performed simultaneously with the experiments in Fig. 4A and Fig. 6, A and B, but are presented in a separate graph for convenience of interpretation.

We initially tested the impact of Y109F IRF2 overexpression alone on the NF1-cis element in the nf1TATACAT reporter construct. In these studies, we found significantly less reporter expression in transfectants with Y109F IRF2 in comparison with wild type IRF2 with and without differentiation (p ≤ 0.002, n = 6). We hypothesize that this is because of the inability of overexpressed Y109F IRF2 to interact with endogenous DNA-bound PU.1. This results in an inability to recruit ICSBP/IRF8. Therefore, we next tested the impact of mutating the conserved IRF2 IRF domain tyrosine on cooperation with overexpressed PU.1. We found that Y109F IRF2 + PU.1 induces significantly less reporter expression from the NF1-cis element-containing construct than wild type IRF2 + PU.1 both in untreated and IFN-γ-treated transfectants (p < 0.002, n = 6). Consistent with the hypothesis that overexpressed Y109F IRF2 is unable to interact with overexpressed PU.1, there is no difference in reporter activity in transfectants with PU.1 + Y109F IRF2 and with PU.1 alone (p = 0.64, n = 3 for undifferentiated transfectants and p = 0.25, n = 3 in IFN-γ-treated transfectants).

If ICSBP/IRF8 interaction with the NF1-cis element requires pre-binding of PU.1 and IRF2, overexpressed Y109F IRF2 would not be anticipated to increase activation of the NF1-cis element by overexpressed ICSBP/IRF8. Consistent with this, we found that activation of the NF1-cis element in transfectants overexpressing Y109F IRF2 + ICSBP/IRF8 is not significantly different from reporter activity in transfectants overexpressing ICSBP/IRF8 alone (p = 0.83, n = 3 for undifferentiated transfectants and p = 0.69, n = 3 for IFN-γ-treated transfectants). This result suggests ICSBP/IRF8 is interacting with only endogenous IRF2 in transfectants overexpressing this IRF2 mutant.

We next investigated the effect on activity of the NF1-cis element of combining Y109F IRF2 with both PU.1 and ICSBP/IRF8. We found significantly less reporter expression in transfections with Y109F IRF2 + PU.1 + ICSBP in comparison with wild type IRF2 and these other two proteins (p ≤ 0.03, n = 6, with and without IFNγ). These results suggest that Tyr-109 in IRF2 is necessary for the overexpressed protein to interact with PU.1. This heterodimer recruits ICSBP/IRF8 and activates transcription.

In control experiments, overexpression of these proteins did not significantly alter reporter expression from the empty minimal promoter control vector. In additional control experiments, we demonstrate that expression of wild type and Y109F IRF2 is equivalent in U937 transfections (Fig. 7B).

Mutation of a Conserved IRF Domain Tyrosine Prevents ICSBP from Activating NF1 Transcription—In our studies above, we found that Tyr-95 in ICSBP/IRF8 is necessary for interaction with the DNA-bound PU.1-IRF2 heterodimer. Also, we previously found that phosphorylation of ICSBP/IRF8 Tyr-95 is essential for activation of NF1-cis element in U937 transfections (15). Therefore, we investigated the role of ICSBP/IRF8-tyrosine phosphorylation in functional interaction with PU.1, IRF2, and the NF1-cis element. These reporter gene assays were performed simultaneously with the experiments in Fig. 4A, Fig. 6, A and B, and Fig. 7A but are presented in a separate graph for convenience of interpretation.

For these studies, U937 cells were co-transfected with the NF1-cis element-containing reporter construct (nf1TATACAT) or control pTATACAT and various combinations of vectors to overexpress wild type or Y95F ICSBP/IRF8, IRF2, and PU.1 (or empty expression vector control) (Fig. 7C). In initial studies, we confirm that Y95F ICSBP/IRF8 induces significantly less reporter activity from the NF1-cis element-containing construct than wild type ICSBP, which is consistent with our previous results (15) (p ≤ 0.01, n = 6 for transfectants with and without IFNγ).

Therefore, we co-overexpressed PU.1 and wild type versus Y95F ICSBP/IRF8. We found that overexpressed PU.1 cooperates significantly less efficiently with overexpressed Y95F ICSBP/IRF8 than with wild type ICSBP/IRF8 in either untreated or IFN-γ-differentiated transfectants (p ≤ 0.01, n = 4). We hypothesize that the interaction of overexpressed PU.1 + ICSBP/IRF8 with the NF1-cis element involves interaction with endogenous IRF2. Because Tyr-95 ICSBP/IRF8 is required for interaction with PU.1 + IRF2, we anticipate that nf1TATACAT reporter activity would be the same with overexpressed PU.1 as with PU.1 + Y95F ICSBP/IRF8. Indeed, there is no difference in reporter activity in these transfectants (p = 0.16, n = 3 for undifferentiated and p = 0.46, n = 3 for differentiated transfectants).
FIGURE 7. Conserved tyrosine residues in the IRF domains of IRF2 and ICSBP/IRF8 are necessary for activation of the NF1-cis element. 

A, overexpressed Y109F IRF2 does not cooperate with PU.1 and ICSBP/IRF8 to activate the NF1-cis element. U937 cells were co-transfected with an artificial promoter construct with multiple copies of the NF1-cis element (nf1TATACT) or empty vector control (pTATACAT) and various combinations of vectors to overexpress Y109F or wild type IRF2 and PU.1 and ICSBP/IRF8. Reporter gene assays were performed with or without IFNγ treatment of the transfectants. Y109F IRF2 induces less reporter expression from nf1TATACT than wild type IRF2 alone or in combination with PU.1, ICSBP/IRF8, or PU.1 plus ICSBP/IRF8 and with and without IFNγ differentiation. In contrast, none of these proteins significantly influenced reporter expression from control pTATACAT.

B, wild type and Y109F IRF2 are equivalently overexpressed in U937 cells. U937 cells were transfected with a vector to overexpress wild type or Y109F IRF2 (or empty vector control). Lysate proteins were separated by SDS-PAGE and serially probed with antibodies to IRF2 and actin (to control for loading). WB, Western blot.

C, overexpressed Y95F ICSBP/IRF8 does not cooperate with PU.1 and IRF2 to activate the NF1-cis element. U937 cells were co-transfected with an artificial promoter construct with multiple copies of the NF1-cis element (nf1TATACT) or empty vector control (pTATACAT) and various combinations of vectors to overexpress Y95F or wild type ICSBP/IRF8 and PU.1 and IRF2. Reporter gene assays were performed on transfectants with or without IFNγ treatment. Y95F ICSBP/IRF8 induces less reporter expression from nf1TATACT than wild type ICSBP/IRF8 alone or with PU.1, IRF2, or PU.1 plus IRF2 and with and without IFNγ differentiation. In contrast, none of these proteins significantly influenced reporter expression from control pTATACAT.
ICSBP/IRF8, IRF2, and PU.1 Activate Nf1 Expression

Therefore, we next tested the impact of ICSBP/IRF8 Tyr-95 phosphorylation on interaction with IRF2. We found that reporter expression from the NF1-cis element-containing constructs is not significantly different in transfectants overexpressing IRF2 + Y95F ICSBP/IRF8 in comparison with transfectants overexpressing IRF2 only (p = 0.1, n = 3 for both undifferentiated and differentiated U937 transfectants). Based on these results, we tested the impact of overexpressed Y95F ICSBP/IRF8 on cooperation with overexpressed PU.1. We found expression of Y95F ICSBP/IRF8 + PU.1 + IRF2 induces significantly less expression from the NF1-cis element-containing construct than ICSBP/IRF8 + PU.1 + IRF2 in both untreated and differentiated transfectants (p = 0.01, n = 4).

In control experiments, none of these combinations of overexpressed proteins influenced expression of the empty minimal promoter/reporter vector (pTATACAT). In previous control experiments, we demonstrated that wild type and Y95F ICSBP is equivalently overexpressed in U937 cells using this vector (15, 16).

PU.1 Is Serine-phosphorylated during IFNγ Differentiation of U937 Cells—If PU.1 is serine-phosphorylated in response to cytokine stimulation, this would provide a mechanism for increased NF1 transcription during myeloid differentiation. Initially, we determined the impact of IFNγ differentiation on PU.1 abundance in U937 cells (Fig. 8A). We found that PU.1 protein increases with IFNγ treatment, and there is a relative increase in a higher molecular weight immunoreactive band. This higher molecular weight band, which is readily detected on 20% acrylamide gels (but not lower % gels), might represent phosphorylated PU.1. The blot was stripped and re-probed for total ERK protein as a loading control and phospho-ERK as a control for IFNγ signaling.

Therefore, we determined the impact of IFNγ treatment on PU.1 phosphorylation in U937 cells. U937 cells were incubated for 48 h with or without IFNγ, followed by metabolic labeling with [32P]orthophosphate. Cell lysates were immunoprecipitated under denaturing conditions with an antibody to PU.1 (or control antibody), separated by SDS-PAGE, and visualized by autoradiography. We found that IFNγ differentiation of U937 cells increases PU.1 phosphorylation (Fig. 8B). This study shows multiple phospho-bands, suggesting multiple phosphorylated forms of PU.1 in these cells.

To determine whether these phospho-residues are serine/threonine versus tyrosine, nuclear proteins from U937 cells were immunoprecipitated under denaturing conditions with an antibody to PU.1 (or control irrelevant antibody). Immunoprecipitates were separated by SDS-PAGE (7.5% acrylamide), and Western blots were serially probed with antibody to phospho-tyrosine and PU.1 (Fig. 8C). We were not able to detect tyrosine-phosphorylated PU.1 by using this approach, consistent with previous results (28). PU.1 appears as a single band in this study because of the percent of acrylamide in the SDS-PAGE.

FIGURE 8. PU.1 is serine/threonine-phosphorylated and IRF2 is tyrosine-phosphorylated during IFNγ differentiation of U937 cells. A, IFNγ differentiation of U937 cells increases PU.1 abundance but not abundance of IRF2. Nuclear proteins were isolated from U937 cells before and after differentiation with IFNγ for 48 h. Proteins were separated on SDS-PAGE, and Western blots (WB) were serially probed with antibodies to PU.1 and IRF2. Blots were also probed with antibody to phospho-ERK as a control for IFNγ treatment and with total ERK as a loading control. Abundance of PU.1 increases with IFNγ treatment of the cells, associated with the appearance of a dominant higher molecular weight band, representing phosphorylated forms of PU.1. In contrast, nuclear IRF2 abundance is not altered by differentiation of the U937 cells. B, IFNγ differentiation of U937 cells increases PU.1 phosphorylation. U937 cells were incubated for 0, 24, or 48 h with IFNγ. Cells were metabolically labeled with [32P]orthophosphate and cell lysates immunoprecipitated (IP) under denaturing conditions with antibody to PU.1 or irrelevant control antibody. Immune-precipitates were separated by SDS-PAGE and phosphoproteins identified by autoradiography. Multiple phospho-forms of PU.1 are identified in IFNγ-treated U937 cells. C, IFNγ differentiation of U937 cells does not induce tyrosine phosphorylation of PU.1. Nuclear proteins were isolated from U937 cells with or without 48 h of IFNγ treatment. Proteins were immunoprecipitated under denaturing conditions with antibody to PU.1 (or control antibody). Immunoprecipitates were separated by SDS-PAGE and Western blots were serially probed with antibody to phospho-tyrosine and PU.1. Although PU.1 abundance increases slightly with IFNγ-treated cells, no tyrosine-phosphorylated PU.1 is detected by this technique. D, IFNγ differentiation of U937 cells increases IRF2-tyrosine phosphorylation. U937 nuclear proteins were isolated from cells with or without 48 h of IFNγ treatment. Proteins were immunoprecipitated under denaturing conditions with an antibody to IRF2 (or control antibody). Immunoprecipitates were separated by SDS-PAGE and Western blots were serially probed with antibodies to phospho-tyrosine and IRF2. Although total IRF2 protein does not increase, the abundance of phospho-IRF2 is increased by IFNγ treatment of U937 cells.

IRF2 Is Tyrosine-phosphorylated during IFNγ Differentiation of U937 Cells—In previous studies, we found that IRF1 and ICSBP/IRF8 become tyrosine-phosphorylated during myeloid differentiation. If IRF2 is similarly modified, this might provide an additional mechanism for cytokine-induced NF1 expression in differentiating myeloid cells. Therefore, we determined the tyrosine phosphorylation state of IRF2 in IFNγ-treated U937 cells. We initially investigated nuclear abundance of IRF2 in differentiating U937 cells (Fig. 8A). We found that total IRF2 protein is not significantly altered by IFNγ treatment.

To determine the tyrosine phosphorylation state of IRF2 in these cells, U937 nuclear proteins were immunoprecipitated under denaturing conditions with an antibody to IRF2 (or control antibody). Immunoprecipitates were separated by SDS-
sine phosphorylation increases during IFNγ differentiation.

**DISCUSSION**

In previous studies, we determined that NF1 activity during myelopoiesis is at least partly regulated by NF1 transcription (4, 15). We also found that activation of the NF1 gene by hematopoietic cytokines is due in part to phosphorylation of a conserved tyrosine residue in the ICSBP/IRF8 IRF domain (15). In these studies, we determine that PU.1 binds to the NF1-cis element and recruits IRF2. We found that binding of this heterodimer is necessary for interaction of ICSBP/IRF8 with the NF1-cis element. Assembly of this activation complex also requires post-translational modification of all three proteins. Because PU.1 and IRF2 undergo post-translational modification during myelopoiesis, our studies identify an additional mechanism that regulates NF1 transcription in differentiating myeloid cells.

In this study, we note that the NF1-cis element is homologous to composite ets/IRF elements in the CYBB and NCF2 genes. However, we found that there are differences in protein binding to the NF1-cis element in comparison with the homologous cis elements in these oxidase genes. The CYBB- and NCF2-cis elements bind PU.1 and IRF1 in undifferentiated myeloid cells (16, 21, 22). This interaction provides basal transcription at early stages of differentiation (21). During terminal differentiation, the PU.1-IRF1 heterodimer recruits ICSBP/IRF8 and the CREB-binding protein to the CYBB and NCF2 promoters (22). Assembly of this complex requires phosphorylation of PU.1 Ser-148 and of the conserved tyrosine residue (Tyr-95) in the ICSBP/IRF8 IRF domain (16).

In contrast, we found no equivalent activation complex binding the NF1-cis element in undifferentiated myeloid cells. In response to differentiating cytokines, an activation complex assembles on the NF1-cis element. Similar to the CYBB- and NCF2-cis elements, assembly of this complex requires initial binding of PU.1. We found that PU.1 recruits IRF2 to the NF1 promoter in a manner that requires phosphorylation of PU.1 serine residues 132 and 133. This interaction also requires the conserved IRF domain tyrosine in IRF2 (Tyr-109). Recruitment of ICSBP/IRF8 to the NF1-cis element by the PU.1/IRF2 heterodimer requires phosphorylation of the conserved tyrosine residue in the ICSBP IRF domain. This provides a specific molecular mechanism for our previous observations of the functional significance of this Tyr residue (15). We also find that recruitment of ICSBP to the NF1-cis element by the PU.1/IRF2 heterodimer requires phosphorylation of serine 148 in PU.1.

These results suggest different residues in PU.1 are responsible for interaction with different IRF proteins. If these residues were substrates for different kinases (or phosphatases), differential signal-dependent events might regulate the interaction of PU.1 with various protein partners. This could specify PU.1 target gene activation patterns at various stages of differentiation or during the inflammatory response. Additionally, these results suggest there are differences in protein/protein/DNA interactions between different ets/IRF-cis elements.

**ICSBP/IRF8, IRF2, and PU.1 Activate NF1 Expression**

**Activation complex binding to the NF1 promoter**

**Differentiated myeloid cells**

![Composite Ets/IRF sequence](image)

**Activation complex binding to the CYBB or NCF2 promoter**

**Differentiated myeloid cells**

![Composite Ets/IRF sequence](image)

Previous studies found that PU.1 and IRF4 bind to a composite ets/IRF sequence in the immunoglobulin K gene (referred to as the K3'E) (29). This interaction requires PU.1 Ser-148 and activates transcription in differentiating B-cells. Similar to the CYBB-, NCF2-, and NF1-cis elements, recruitment of the IRF protein to the K3'E requires PU.1 binding. For this cis element, the minimal PU.1-consensus is 3'-GGAA-5', consistent with our present results (30). Crystallography studies have been performed to obtain additional information regarding protein interaction with composite ets/IRF sequences. In these studies, the PU.1 ets domain and the IRF4 IRF domain were co-crystallized with the K3'E. These studies indicate that the PU.1 PEST domain is sterically available for protein/protein interactions (31). These studies also indicate that the conserved IRF domain Tyr residue in IRF4 is positioned to interact with the PU.1 ets domain. Therefore, these crystallography results are not inconsistent with our model for PU.1/IRF protein interactions.

Based on our studies, we anticipate that interaction of DNA-bound PU.1 with one IRF protein creates a binding site for a second IRF protein. We also anticipate that binding of this second IRF protein involves contact with PU.1 and the first IRF (Fig. 9). This conclusion is based on DNA affinity purification studies and on assays in which PU.1 interaction with ICSBP/IRF8 or IRF2 was studied in the absence of DNA binding. In these studies, PU.1 interaction with ICSBP/IRF8 requires PU.1 Ser-148 and PU.1 interaction with IRF2 requires PU.1 Ser-132/133.

We confirmed the model generated from in vitro binding assays with functional assays using transfection of U937 cells. We found that overexpression of these two IRF proteins has a more than additive impact on the NF1-cis element, indicating nonredundant roles for IRF2 and ICSBP/IRF8. These studies also indicate the functional importance of phosphorylation of Ser-132, -133, and -148 of PU.1, Tyr-109 of IRF2, and Tyr-95 of ICSBP/IRF8 for NF1 transcription. These results are consistent with the role of post-translational modification of these pro-
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teins in regulating Nf1 expression in differentiating myeloid cells.

PU.1 is a key transcription factor in myelopoiesis. A number of previously identified PU.1 target genes confer mature phagocyte functional competence (11, 21, 22, 32–36). This is consistent with immunodeficiency found in PU.1-deficient murine models (37, 38). However, mutations in the gene encoding PU.1 have been found in human AML (39, 40). Additionally, studies of leukemia in γ-irradiated mice documented frequent deletion in the PLI gene (41). These results suggest a possible role for PU.1 in leukemogenesis. To investigate this, other investigators generated a conditional PU.1-knock-out mouse (42). These animals develop a myeloproliferative disorder that evolves to AML over several months. These results suggest that PU.1 function is partly regulated by protein abundance. Consistent with this, PU.1 is expressed at low levels in hematopoietic stem cells and is relatively more abundant during monocyte versus neutrophil differentiation (43). However, target genes responsible for dysregulated myeloproliferation in PU.1-deficient cells had not been identified prior to this study.

The role of IRF2 in myelopoiesis and leukemogenesis is less clear. Relatively few IRF2 target genes have been identified in myeloid cells. Previous studies found IRF2 activates transcription of some genes involved in the innate immune response (23, 44–46). IRF2 also activates the gene encoding histone H4 during G1/S transition (5). However, no IRF2 target genes that regulate cellular proliferation or cell cycle progression have been identified previously. Identification of NFI as IRF2 target genes suggests that IRF2 deficiency might be expected to result in cytokine hypersensitivity. However, IRF2-deficient mice do not exhibit a myeloproliferative disorder (47). It is possible that IRF2 is functionally redundant with another IRF protein for regulation of genes involved in myeloproliferation. Alternatively, it is possible that IRF2 impacts target genes that both up- and down-regulate proliferation. If so, the net impact of IRF2 might reflect the protein/protein or protein/DNA interactions in which it participates under a given set of conditions.

Therefore, these studies determine that cytokine-induced NFI transcription involves phosphorylation-dependent assembly of an activation complex that includes PU.1, IRF2, and ICSBP/IRF8. These studies provide a mechanism by which both PU.1 and IRF2 influence proliferation in differentiating myeloid cells. Clarification of such molecular mechanisms may suggest rational therapeutic targets for malignant myeloid disorders.

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