PU.1, Interferon Regulatory Factor 1, and Interferon Consensus Sequence-binding Protein Cooperate to Increase gp91<sub>phox</sub> Expression*

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gp91<sub>phox</sub> is a subunit of the phagocyte respiratory burst oxidase catalytic unit. Transcription of CYBB, the gene encoding gp91<sub>phox</sub>, is restricted to terminally differentiated phagocytic cells. An element in the proximal CYBB promoter binds a protein complex, referred to as hematopoiesis-associated factor (HAF1), that is necessary for interferon-γ (IFNγ)-induced gp91<sub>phox</sub> expression. In these investigations, we determined that HAF1 was a multiprotein complex, cross-immunoreactive with the transcription factors PU.1, interferon regulatory factor 1 (IRF-1), and interferon consensus sequence-binding protein (ICSBP). In electrophoretic mobility shift assay, the HAF1 complex was reconstituted by either in vitro translated PU.1 with IRF-1 or PU.1 with ICSBP, but not by IRF-1 with ICSBP. HAF1a, a slower mobility complex with the same binding site specificity as HAF1, was also investigated. Similar to the HAF1 complex, the HAF1a complex was cross-immunoreactive with PU.1, IRF-1, and ICSBP. Unlike the HAF1 complex, reconstitution of the HAF1a complex required in vitro translated PU.1 with both IRF-1 and ICSBP. An artificial promoter construct containing the HAF1/HAF1a binding site was modestly activated in the myelomonocytic cell line U937 by co-transfection either with PU.1 and IRF-1 or with PU.1 and ICSBP, but it was strongly activated by co-transfection with PU.1, IRF-1, and ICSBP. This activation required serine 148-phosphorylated PU.1. These studies describe a novel mechanism for PU.1 transcriptional activation via interaction with both IRF-1 and ICSBP, a target gene for the interaction of IRF-1 with ICSBP, and a novel activation function for ICSBP as a component of a multiprotein complex.

During hematopoiesis, transcription of lineage-restricted genes contributes to development of the various blood cell lineages. Although erythropoiesis is regulated by lineage-restricted transcription factors (1), many of the transcription factors that regulate myelopoiesis, such as ETS proteins and interferon regulatory factor (IRF) proteins, are more broadly expressed (2–4). The ETS transcription factor PU.1, which is expressed exclusively in myeloid and B-cells, regulates the expression of a number of genes common to both of these lineages, including the genes encoding CD18 (2), and the major histocompatibility complex I-β (5). However, PU.1 is also essential for transcription of genes expressed in myeloid cells but not B-cells, including the gene encoding the macrophage scavenger receptor (3), the gene encoding CD11b (6), and NCF1, which encodes p47<sub>phox</sub> (7). Also, PU.1 has been implicated in transcription of the B-cell-specific genes encoding the immunoglobulin κ light chain (8), J chain (9), λ 2–4 enhancer (10), and μ heavy chain (11). Therefore, myeloid versus B-cell-specific regulation of genes by PU.1 must involve additional mechanisms such as lineage-specific partners and/or lineage-specific protein modification.

The IRF proteins have been postulated to be involved in transcriptional regulation of genes involved in the immune response. Post-translational modification and partnering have been postulated as mechanisms involved in regulation by IRF proteins (12, 13). The ubiquitously expressed interferon regulatory factor 1 (IRF-1) (12) has been demonstrated to physically interact with the myeloid and B-cell-specific interferon consensus sequence-binding protein (ICSBP) (12, 13). This interaction is governed by interferon-induced phosphorylation of both proteins and is postulated to provide a mechanism for activation of genes during the inflammatory response (13). However, the significance of this interaction in lineage-specific gene expression is hypothetical, since no target genes have been described.

In these investigations, we explored the involvement of ETS and IRF proteins in transcription of the myeloid-specific CYBB gene, which encodes gp91<sub>phox</sub>, the heavy chain of the phagocyte respiratory burst oxidase (14–16). The expression of gp91<sub>phox</sub> is limited to phagocytic cells that have matured beyond the promyelocyte stage and continues until cell death (17). CYBB transcription is therefore both lineage- and differentiation state-specific. In mature phagocytes, the expression of gp91<sub>phox</sub> protein leads to chronic granulomatous disease (CGD), a disorder of host defense (19).

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‡ The abbreviations used are: IRF, interferon regulatory factor; CGD, chronic granulomatous disease; IFNγ, interferon-γ; bp, base pair; EMSA, electrophoretic mobility shift assay; PIP, PU.1-interacting protein; ICSBP, interferon consensus sequence-binding protein; HAF1, hematopoiesis-associated factor 1; ISRE, interferon-stimulated response element; κ3E, κ<sub>2</sub>' enhancer element; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis.
Investigation of two kindreds with CGD revealed a cis element in the proximal CYBB promoter that was necessary for lineage-specific, IFN-γ-induced gp91phox expression (20, 21). In the affected individuals of these two kindreds, normal gp91phox protein was absent (21), and genomic DNA analysis revealed a single bp mutation at either −55 or −57 bp of the CYBB gene (21). By EMSA, either of these mutations disrupted binding of a specific protein complex to the CYBB 5′-flank (20, 21). Since binding of this complex was manifest only with nuclear proteins isolated from cells of hematopoietic lineages (20), it was referred to as the hematopoeisis-associated factor 1 (HAF1) complex. Linked CYBB promoter-reporter constructs containing the proximal 450 bp of the CYBB 5′-flank were transfected into myeloid cell lines, and the transfectants were differentiated with IFNγ. Wild type promoter constructs, but not CGD mutant promoter constructs, had IFN-γ-inducible reporter gene expression (20). No IFN-γ-inducible reporter gene expression was demonstrated when the 450-bp CYBB promoter constructs were transfected into the epithelial cell line, HeLa (20).

In this study, we investigated the components of the HAF1 complex. The sequence of the HAF1 binding site (20) included an ETS binding consensus sequence (−50 to −55 bp; 5′-GAG-GAA-3′) (4, 22), immediately adjacent to a sequence with homology to the IRF binding consensus sequence (−53 to −63 bp; 5′-GAA TGAAAAC-3′) (4, 23) (both on the noncoding strand). This combination is reminiscent of the immunoglobulin κ 3′-enhancer element (κE3′) (8). The κE3′ is a positive cis element that binds PU.1 as a heterodimer with a lymphoid-specific, IRF protein referred to as PU.1-interacting protein (PIP) (8, 24).

However, no similar interactions between PU.1 and IRF family members have been documented in myeloid gene regulation. In these studies, we investigated the presence of PU.1 and IRF family members in the HAF1 complex and the action of PU.1 and IRF family members on the CYBB HAF1 binding element.

**EXPERIMENTAL PROCEDURES**

**Plasmids**

**Protein Expression Constructs and cDNAs—**The cDNA for human PU.1 was obtained from M. Klemm (Indiana University, Indianapolis, IN) and subcloned into the mammalian expression vector pSRα (25) (obtained from T. Gabig, Indiana University), the pBluescript vector for in vitro transcription and translation (Stratagene, La Jolla, CA), and the pGEX2 vector for fusion protein expression (Amersham Pharmacia Biotech). The cDNA for murine PU.1 and the serine to alanine mutants of murine PU.1 at serines 146, 146, serines 132 and 133, and serines 41 and 45 were obtained from M. Atchison (University of Pennsylvania, Philadelphia, PA) and subcloned into pSRα. The human ICSBP cDNA was obtained from B-Z. Levi (Technicon, Haifa, Israel) and subcloned into the mammalian expression vector pcDNAamp. The human FLI-1 cDNA was obtained from the University of Alabama at Birmingham, or in the laboratory of Dr. B. Wells Center for Pediatric Research at Indiana University, in the laboratory of Dr. Donald Miller at the University of Alabama at Birmingham. CYBB promoter sequence (4) oligonucleotides used were as follows: CYBB promoter −32 to −69 bp, 5′-CTGTGTTCGTTCCCTTCTGTTGAGAAGAGCATAG-3′; CGD mutant promoter −32 to −69 bp (−57) (21), 5′-CTGTGTTCGTTCCCTTCTGTTGAGAAGAGCATAG-3′; CGD mutant promoter −32 to −69 (−55) (21), 5′-CTGTGTTCGTTCCCTGTTGAGAAGAGCATAG-3′; E74 (22), 5′-AAATAACCGGAAATCGTTGGATGTA-3′; Pu/PuP binding site from the immunogloblin κE3′ (8), 5′-CTTTGNNGaAGCTTGGACAAAGCATCA-3′; GASGBP (γ-interferon activation sequence of the guanylate-binding protein) (28), 5′-AGTTTCA-TATACCTCTATATC-3′; CYBB promoter −214 to −243 bp (IFR-2 binding site) (29), 5′-AGAAAAATTTTCATTTTCAAGCTTATGTTGACGTGGATCATTAT-3′.

**Cell Culture and Stable Transfections**

All cell lines were of human origin. The epithelial carcinoma line HeLa (20) was obtained from ATCC (Rockville, MD). The promyelocytic leukemia cell line PLB985 (31) was obtained by Thomas Rado (University of Alabama at Birmingham). The promyelocytic leukemia cell line U937 (32) was obtained from Andrew Kraft (University of Colorado, Denver, CO). Cell lines were maintained and differentiated as described previously (20). PLB985 and U937 cells were treated with human recombinant IFNγ (Boehringer Mannheim) at 100 units/ml for various times. HeLa cells were treated with IFNγ at 50 units/ml for 24 h.

**In Vitro Translation of Proteins**

In vitro transcription and translation of human and murine PU.1, ICGBP, IRF-1, IRF-2, STAT91, and TF1phox sequences cloned into pBluescript or pcDNAaamp was performed using the in vitro transcription and rabbit reticulocyte lysate in vitro translation systems by Promega according to the manufacturer's instructions (Promega, Madison, WI).

**Electrophoretic Mobility Shift Assays**

Nuclear extract proteins were prepared from tissue culture cells growing at log phase by the method of Dignam (33) with protease inhibitors as described (20). Protein assays were performed by the method of Lowry (34).

Oligonucleotide probes were prepared from plasmid constructs by restriction digestion and filled in by Klenow fragment with a nucleotide mix containing dATP, dGTP, dTTP (Boehringer Mannheim) and [α-32P]dCTP (Amersham Pharmacia Biotech). Specific activity of the probes was 2×106 cpm/m mole of DNA.

EMSA and antibody supershift assays were performed as described (20). Antiserum to PU.1 was obtained from M. Klemm (Indiana University) (27) and antiserum to FLI-1 was obtained from Alon Bernstein (Mount Sinai Hospital, Ontario, Canada) (22). Anti-IRF-2 and anti-IRF-2 antisera were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-IRF-2 antisera (whole protein) was obtained from Richard Pine (Public Health Research Institute, New York, NY) (35). Anti-IRF-1 serum directed against amino acids 306–325 (nonconserved among IRF family members) was obtained from Santa Cruz Biotechnology, Inc. Anti-ICSBP serum (whole protein) was obtained from Keiko Ozato (National Institutes of Health, Bethesda, MD) (36). Antiserum to peptides in ICSBP nonconserved with other IRF family members (anti-310, anti-311, and anti-312) was obtained from S. Vogel (Armed Forces Institute, Bethesda, MD) (37). Anti-PIP serum was a very generous gift from M. Atchison (University of Pennsylvania, Philadelphia, PA).

**Co-precipitation Assays**

PU.1GEX2 and control pGEX2 in JM109 Escherichia coli were grown to log phase, supplemented to 0.1 mM isopropyl-1-thio-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, 0.5% nonidet P-40, 0.1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, 5 mM NaF) and sonicated.
on ice. Debris was removed by centrifugation, and the lysate was incubated 30 min at 4 °C with glutathione-agarose beads (Sigma) and washed extensively with HN buffer. The beads were preincubated for 30 min at 4 °C with 5 μl of control rabbit reticulocyte lysate and then for 1 h with 20 μl of 35S)methionine-labeled in vitro translated protein and washed extensively in HN buffer. Proteins were eluted with SDS-PAGE sample buffer and separated on 12% SDS-PAGE, and an autoradiograph was performed.

Immunoprecipitation experiments were performed with 200 μg of nuclear proteins at 0.2 μg/ml in HN buffer with protease inhibitors as above. Nuclear proteins were incubated with 5 μl of either preimmune serum or anti-ICSBP serum (310 and 311 at 1:1) or IRF-1 antibody (Shearwater Biotech) as described (38).

To determine whether the ETS consensus CYBB Promoter—binding sites from two other genes (8, 27) but not by oligonucleotide competition method using [3H]chloramphenicol (Amersham Pharmacia Biotech) as described (38). Chloramphenicol acetyltransferase (CAT) assays were performed by the extraction method using [3H]chloramphenicol (Amersham Pharmacia Biotech) as described (38).

Cell extracts were analyzed as above after 24 h of incubation at 37 °C and 5% CO2 in 10 ml of DME, 10% fetal calf serum, 1% penicillin-streptomycin, and a 100-fold molar excess of double-stranded oligonucleotide competitors. Lanes 1 and 2, no competitor; lane 3, homologous sequence; lane 4, −55 bp CYBB promoter sequence mutant; lane 5, −57 bp CYBB promoter sequence mutant; lane 6, PU.1 binding site from the major histocompatibility complex II promoter; lane 7, PU.1 site from the α-globin promoter. The arrow indicates the PU.1 complex. In vitro translated PU.1 binding to the −32 to −69 bp CYBB promoter sequence was recognized by PU.1 antisera. EMSA was performed with the −32 to −69 bp CYBB promoter sequence probe, either control rabbit reticulocyte lysate (lane 1) or in vitro translated PU.1 (lanes 2–8) and a 100-fold molar excess of double-stranded oligonucleotide competitors. Lanes 1 and 2, no competitor; lane 3, homologous sequence; lane 4, −55 bp CYBB promoter sequence mutant; lane 5, −57 bp CYBB promoter sequence mutant; lane 6, PU.1 binding site from the major histocompatibility complex II promoter; lane 7, PU.1 site from the α-globin promoter.

RESULTS
The Transcription Factor PU.1 Interacts with the Proximal CYBB Promoter—To determine whether the ETS consensus sequence in the HAF1 binding site (20) was able to interact with PU.1, EMSA was performed with in vitro translated PU.1 and a double-stranded oligonucleotide probe with the −32 to −69 bp CYBB promoter sequence. In vitro translated PU.1 generated a complex not found with control lysate (Fig. 1). Binding of this complex was competed for by unlabeled homologous oligonucleotide and oligonucleotides containing the PU.1 binding sites from two other genes (8, 27) but not by oligonucleotides representing either of the CYBB CGD kindred mutations (−55 or −57 bp) or an unrelated oligonucleotide (Fig. 1A). The complex generated by in vitro translated PU.1 was recognized by PU.1 antisera but not by rabbit preimmune serum or antisera to the ETS protein FLI-1 (Fig. 1B). EMSA with in vitro translated FLI-1 and the −32 to −69 bp CYBB promoter sequence probe demonstrated no binding, although in vitro translated FLI-1 bound a high affinity FLI-1 binding site (E74; Ref. 22).

The ability of PU.1 in nuclear proteins of myeloid cells to bind to the −32 to −69 bp CYBB promoter sequence was investigated. In previously published experiments (20, 21), synthetic oligonucleotide probes containing the −32 to −69 bp sequence of the CYBB promoter were generated by labeling annealed, single-stranded oligonucleotides. EMSA with those probes had significant nonspecific binding of high mobility protein complexes, perhaps due to protein binding to single-stranded oligonucleotides in the probe. In these experiments, double-stranded synthetic oligonucleotides were subcloned into a plasmid vector, and probes were generated by liberating the CYBB sequence from the vector and labeling the restriction product. EMSA performed with these probes had fewer nonspecific bands in comparison with previous experiments (20, 21).

EMSAs of the −32 to −69 bp CYBB promoter sequence probe with nuclear proteins from the myelomonocytic cell line U937 (with and without IFNγ treatment) demonstrated the presence of a specific, high mobility complex that had been previously obscured (Figs. 1B and 2, A and B). This complex was of similar mobility to that generated using in vitro translated PU.1 (Fig. 1B). Binding of this complex was specifically disrupted by antisera to PU.1 and was competed for by unlabeled homologous oligonucleotide or an oligonucleotide containing the PU.1 sequence probe demonstrated no binding, although
Fig. 2. PU.1 from myeloid nuclear proteins bound the CYBB promoter sequence. A, PU.1 from undifferentiated myeloid cell nuclear proteins bound the CYBB promoter. EMSA was performed with the −32 to −69 bp CYBB promoter sequence probe and nuclear proteins (4 μg) from U937 cells in the presence antibodies or a 100-fold molar excess of double-stranded competitor oligonucleotides. Lane 1, rabbit preimmune serum (3.0 μl); lane 2, PU.1 antiserum (3.0 μl); lane 3, FLI-1 antiserum (3.0 μl); lane 4, ETS domain antibody (amino acids 362–374 of ETS-1) (3.0 μg); lane 5, heterologous oligonucleotide (−331 to −381 bp sequence of the CYBB promoter); lane 6, homologous oligonucleotide; lane 7, κE3′ oligonucleotide; lane 8, α-globin gene promoter CCAAT box oligonucleotide. Upper arrowhead, the HAF1a complex; lower arrowhead, the PU.1 complex; open arrowhead, CP1 complex. The asterisk indicates the variably present proteolytic fragment of HAF1. B, PU.1 from IFNγ-treated myeloid cell nuclear proteins bound to the CYBB promoter. EMSA was performed with the −32 to −69 bp CYBB promoter sequence probe and nuclear proteins (3.0 μg) from U937 cells that had been treated for 48 h with IFNγ in the presence of antisera. Lane 1, rabbit preimmune serum (3.0 μl); lane 2, anti-PU.1 serum (3.0 μl); lane 3, anti-FLI-1 (3.0 μl); lane 4, anti-ETS domain (3.0 μg); lane 5, no serum. Upper arrowhead, the HAF1a complex; lower arrowhead, the HAF1 complex; upper arrow, the CP1 complex; lower arrow, the PU.1 complex. The asterisk indicates the variably present proteolytic product of HAF1. C, binding of the HAF1 and HAF1a complexes had similar lineage and differentiation state distribution. EMSA was performed with the −32 to −69 bp CYBB promoter sequence probe in the presence of various nuclear proteins (2.0 μg). Lane 1, nuclear proteins from U937 cells; lane 2, nuclear proteins from U937 cells treated with IFNγ; lane 3, nuclear proteins from HeLa cells; lane 4, nuclear proteins from HeLa cells treated with IFNγ. Upper arrow, the HAF1a complex; lower arrow, the HAF1 complex; arrowhead, the CP1 complex. The asterisk indicates a partial proteolytic fragment of HAF1. D, binding of PU.1, HAF1, and HAF1a to the CYBB promoter was abolished by the CGD promoter mutations. EMSA was performed with the wild type −32 to −69 bp CYBB promoter sequence probe (lanes 1) or a −57 bp mutant probe (lanes 2) with nuclear proteins (1.0 μg) from U937 cells treated for 48 h with IFNγ. Upper arrowhead, the HAF1a complex; lower arrowhead, the HAF1 complex; upper arrow, the CP1 complex; lower arrow, PU.1. E, immunodepletion of PU.1 from myeloid cell nuclear proteins abolished binding of the PU.1, HAF1, and HAF1a complexes to the CYBB promoter, and the HAF1 and PU.1 complexes were reconstituted by in vitro translated PU.1. EMSA was performed with the −32 to −69 bp CYBB promoter sequence probe with nuclear proteins (1.0 μg) from U937 cells that had been sham immunodepleted with Staphylococcus protein A beads (lane 1) or PU.1 depleted with anti-PU.1 immunobeads (lanes 2–5) with control rabbit reticulocyte lysate (0.5 μl) (lane 2) or in vitro translated PU.1 (lane 3, 0.5 μl; lane 4, 1.0 μg; lane 5, 2.0 μg). Upper arrowhead, the HAF1a complex; lower arrowhead, the HAF1 complex; upper arrow, CP1; open arrowhead, PU.1; asterisk, a partial translation product of in vitro translated PU.1.

binding site from the κE3′ but not by several unrelated oligonucleotides (Fig. 2A, identical results seen with nuclear proteins from IFNγ-treated U937 cells).

Four protein complexes that bound to the −32 to −69 bp probe in EMSA with U937 nuclear proteins, three in addition to the PU.1-like complex (Fig. 2A). These complexes were not changed by IFNγ treatment of the U937 cells (Fig. 2B). One of the three complexes was the previously described HAF1 com-
plex (12). Similarly to the PU.1-like complex, binding of the HAF1 complex was specifically disrupted by anti-PU.1 serum and was competed for by homologous oligonucleotide and the κ3′-sequence oligonucleotide but not by unrelated oligonucleotides (Fig. 2A). The results of these experiments were identical with nuclear proteins from IFN-γ-treated U937 cells (Fig. 2F) and the myeloid cell line PLB985 (not shown). The HAF1 complex has been previously demonstrated to represent specific protein binding to the −32 to −69 bp sequence (20). There was also a variably present complex of slightly faster mobility than the HAF1 complex, which probably represents partial proteolysis (20).

A lower mobility complex, referred to as HAF1a, had the same pattern of antibody reactivity and binding competition as HAF1 and PU.1 (Fig. 2). Similarly to the HAF1 and PU.1 complexes, HAF1a complex binding was disrupted by either of the CGD promoter mutations (see below). Increasing protein concentration increased HAF1a binding relative to HAF1 binding, suggesting that HAF1a might be a multimeric form of HAF1. Also, similar to the HAF1 complex, binding of the HAF1a complex was unaffected by treatment of the cells with IFN-γ, and binding of the HAF1a complex was not present in EMSA with nuclear proteins from the nonhematopoietic cell line, HeLa (Fig. 2C).

The fourth complex binding to the −32 to −69 bp CYBB promoter sequence probe was previously demonstrated to be the CCAAT factor, CP1, binding the CCAAT box sequence present in the probe (20). This CCAAT box was previously shown to be dispensable for CYBB promoter function (20).

As previously demonstrated, either the −55 or −57 bp CGD mutation abolished binding of the HAF1 complex, but not CP1, in EMSA with myeloid nuclear proteins and the −32 to −69 bp sequence probe (20, 21). These mutations also abolished binding of the HAF1a and PU.1-like complexes, consistent with the shared binding site specificities of HAF1, HAF1a, and PU.1 (Fig. 2D). These results were identical with nuclear proteins from U937 and PLB985 cells with and without 48 h of IFN-γ-induced differentiation.

To further investigate whether PU.1 was a component of the HAF1 complex, nuclear proteins from U937 cells were PU.1 immunodepleted with antisera covalently linked to Staphylococcus protein A beads. PU.1 immunodepleted nuclear proteins and sham depleted nuclear proteins were used in EMSA with the −32 to −69 bp CYBB promoter sequence probe. The HAF1, HAF1a, and PU.1 complexes did not bind to the −32 to −69 bp probe in EMSA using PU.1-depleted nuclear proteins, although these complexes were present using sham depleted nuclear proteins (Fig. 2E). Neither PU.1 nor sham deplet in vitro altered binding of the CP1 complex to the −32 to −69 bp probe. Identical results were obtained using U937 or PLB985 nuclear proteins with or without IFN-γ (data not shown).

To investigate whether HAF1 contained PU.1 or another cross-immunoreactive protein, a reconstitution experiment was performed. PU.1-depleted nuclear proteins were mixed with either in vitro translated PU.1 or control rabbit reticulocyte lysate, and EMSA was performed using the −32 to −69 bp CYBB promoter sequence probe. In vitro translated PU.1, but not control rabbit reticulocyte lysate, reconstituted the PU.1 complex and the HAF1 complex (Fig. 2E). The HAF1a complex was not reconstituted, even with increasing amounts of in vitro translated PU.1, suggesting that PU.1 was not the limiting factor for reconstitution of this complex. Since HAF1a binding requires higher protein concentration than does HAF1, co-immunoprecipitation of other protein(s) with PU.1 might leave adequate amounts of these co-precipitating proteins to reconstitute HAF1, but not HAF1a. This possibility is addressed below.

Interferon Regulatory Factors IRF-1 and ICSBP Interact with the Proximal CYBB Promoter—Similarly to the PU.1/PIP binding site in the immunoglobulin κ gene 3′-enhancer element, the −32 to −69 bp sequence of the CYBB promoter also contained a PU.1 binding site overlapping an ISRE (23). Since factors of the IRF and STAT families bind to ISRE sequences, the possibility that HAF1 might be composed of PU.1 with PIP or another IRF, or else a STAT, was explored.

EMSA was performed using the −32 to −69 bp CYBB promoter probe, nuclear proteins from U937 cells with and without IFN-γ differentiation, and various antibodies. Neither preimmune serum nor antisera raised against IRF-2, PIP, or ISGF3γ (p48) nor the STAT proteins of the ISGF3 complex (using an antibody that recognized both p91(STAT1α) and p86) had any effect upon the HAF1 or HAF1a complexes (Fig. 3A). Control experiments demonstrated that these antisera could recognize protein complexes interacting with previously described DNA-binding sites for these proteins (data not shown) (8, 28, 29).

In EMSA using the −32 to −69 bp CYBB promoter sequence with nuclear proteins from U937 cells (with or without IFN-γ treatment), binding of the HAF1 complex was partly disrupted by antisera to either IRF-1 or ICSBP (Fig. 3A). Complete disruption of the HAF1 complex was demonstrated in the presence of both anti-IRF-1 and anti-ICSBP sera (Fig. 3B). In contrast to the partial disruption of the HAF1 complex by antisera to either IRF-1 or ICSBP, the HAF1a complex was completely disrupted by either the IRF-1 or the ICSBP anti-serum (Fig. 3A). These complexes were disrupted by anti-IRF-1 serum raised to the whole protein (35) and to a peptide in the region of the protein most divergent from other IRF family members. Similarly, the HAF1 and HAF1a complexes were disrupted by anti-ICSBP serum raised to the whole protein (36) and to peptides that were divergent from other IRFs (37).

In vitro translated IRF-1, ICSBP, and PU.1 were used, in various combinations, in EMSA of the −32 to −69 bp CYBB promoter sequence. Unlike in vitro translated PU.1, neither in vitro translated IRF-1 nor ICSBP was able to bind to the −32 to −69 bp probe (Fig. 3C). Both of these in vitro translated proteins were able to bind to high affinity binding sites for these proteins in control experiments (not shown). However, the combination of PU.1 with either IRF-1 or ICSBP generated a complex of the same mobility as HAF1 but did not generate a complex of the same mobility as HAF1a (Fig. 3C). No complex was generated by the combination of IRF-1 with ICSBP in the absence of PU.1. Only binding reactions that included all three proteins, PU.1, IRF-1, and ICSBP, generated complexes of the same mobility as both HAF1 and HAF1a.

To determine whether there was direct interaction between PU.1 and IRF proteins, in vitro translated IRFs were used in co-precipitation experiments with PU.1 expressed as a glutathione S-transferase fusion protein (PU.1/GST). PU.1/GST, prebound to glutathione-agarose beads, co-precipitated IRF-1, ICSBP, and PIP, but not STAT1α (36) (Fig. 4A). Similarly, antisera against either IRF-1 or ICSBP co-immunoprecipitated PU.1 from U937 cell nuclear proteins by Western blot (Fig. 4B). Therefore, in the experiments presented in Fig. 2E, co-immunodepletion of ICSBP and/or IRF-1 with PU.1 may have left inadequate amounts of one or both of these proteins to reconstitute the HAF1a complex.

Interaction of PU.1 with a CYBB Promoter Element Increases Transcription—To determine the effect of PU.1 upon the CYBB promoter, U937 cells were co-transfected with an expression vector containing the PU.1 cDNA (PU.1/pSRE) and an artificial promoter construct. The artificial promoter constructs included either four copies of the wild type −32 to −69 bp CYBB pro-
The interferon regulatory factors IRF-1 and ICSBP were incubated with antisera (1.0 μl of 1:10 dilution), IRF-1, and ICSBP (0.2 μl) and control reticulocyte lysate (1.0 μl). Each experiment was repeated six times.

Absolute CAT activity of transfected p-TATACAT was not significantly different than either p-ha1:TATACAT or p-cgdTATACAT (−5.7 ± 2.3%, n = 6; not shown). Co-transfection of PU.1/pSREs with p-ha1:TATACAT resulted in a modest increase in CAT activity (Fig. 5A) that was not demonstrated with co-transfection of PU.1 and p-cgdTATACAT (−4.1 ± 2.3%, n = 6; not shown).

Co-transfection with control plasmid pSREs had no effect on absolute CAT activity of any of the three reporter constructs. Incubation of p-TATACAT or p-ha1:TATACAT transfected with IFNγ for 24 h did not change absolute CAT activity of either of the constructs, and this result was not affected by co-transfection of PU.1 with p-TATACAT. However, IFNγ-treated co-transfectants with PU.1 and p-ha1:TATACAT had twice as much CAT activity as in the absence of the cytokine (Fig. 5A). Cells of the epithelial cell line HeLa do not express CAT activity in the absence of the promoter sequence or four copies of the −57 bp mutant sequence, linked to a minimal promoter and a CAT reporter gene. A comparison was made between CAT activity from the control plasmid (p-TATACAT; Ref. 26) and each of the CYBB promoter sequence-containing constructs (p-ha1:TATACAT or p-cgdTATACAT for the wild type and mutant promoter constructs, respectively). Results were expressed as a percentage increase or decrease in CAT activity of the CYBB sequence containing plasmid relative to CAT activity of control p-TATACAT plasmid (therefore, p-TATACAT activity was 0%). Each experiment was repeated six times.

Absolute CAT activity of transfected p-TATACAT was not significantly different than either p-ha1:TATACAT or p-cgdTATACAT (−5.7 ± 2.3%, n = 6; not shown). Co-transfection of PU.1/pSREs with p-ha1:TATACAT resulted in a modest increase in CAT activity (Fig. 5A) that was not demonstrated with co-transfection of PU.1 and p-cgdTATACAT (−4.1 ± 2.3%, n = 6; not shown).
Interaction of IRF-1 and ICSBP with a CYBB Promoter Elements Increase Transcription—To investigate the effect of IRF-1 and ICSBP on the −32 to −69 bp CYBB promoter sequence, U937 cells were co-transfected with an expression vector containing either the cDNA for IRF-1 or ICSBP (IRF-1/pcDNAamp and ICSBP/pcDNAamp, respectively) and either p-TATACAT or p-haf1TATACAT. As above, results are expressed as the percentage increase in absolute CAT activity of p-haf1TATACAT transfectants over the absolute CAT activity of PU.1 or gp91phox. In HeLa cells transfected with the same constructs as above, there was no significant difference in CAT activity between p-TATACAT and p-haf1TATACAT, with or without PU.1 co-transfection (Fig. 5A).

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ity of p-TATACAT transfectants (p-TATACAT activity is 0%). Each experiment was repeated four times. Co-transfection of p-TATACAT with PU.1, IRF-1, or ICSBP, in any combination, had no significant effect on absolute CAT activity (with or without IFNγ).

U937 co-transfectants with p-haf1TATACAT and IRF-1 or ICSBP demonstrated a modest increase in CAT activity that was not affected by 24-h IFNγ incubation (Fig. 5B). Since there is endogenous PU.1 in U937 cells, these experiments did not suggest that either of these proteins was interacting with the promoter sequence in the absence of PU.1.

Transfection of U937 cells with PU.1 and either IRF-1 or ICSBP, or with IRF-1 and ICSBP without PU.1, demonstrated a modest, approximately 2-fold increase in CAT activity of co-transfected p-haf1TATACAT (Fig. 5B). IFNγ incubation did not significantly change CAT activity of co-transfectants with PU.1, IRF-1, and p-haf1TATACAT or IRF-1, ICSBP, and p-haf1TATACAT. However, IFNγ incubation of PU.1, ICSBP, and p-haf1TATACAT transfectants further increased CAT activity approximately 2-fold (Fig. 5B).

In contrast, a 10-fold increase in CAT activity was demonstrated in U937 co-transfectants with p-haf1TATACAT and expression vectors containing PU.1, IRF-1, and ICSBP (relative to p-TATACAT co-transfectants), which was not further augmented by 24 h IFNγ incubation (Fig. 5B). This effect was greater than the calculated additive effects of PU.1 plus IRF-1 and PU.1 plus ICSBP on co-transfected p-haf1TATACAT. IFNγ treatment of U937 cells significantly increases the abundance and phosphorylation of IRF-1 protein but less so of ICSBP (13). Therefore, IFNγ treatment of ICSBP, PU.1, and p-haf1TATACAT co-transfectants partly duplicated the effect of co-transfection of IRF-1 with ICSBP, PU.1, and p-haf1TATACAT.

To determine whether PU.1, IRF-1, and ICSBP were functionally interacting with each other through one HAF binding site or if the proteins were interacting between the multimerized sites in the p-haf1TATACAT construct, the U937 transfections were repeated with a construct containing one copy of the −32 to −69 bp CYBB promoter sequence. CAT activity of single binding site construct transfected was not significantly different than p-TATACAT for co-transfectants with PU.1, IRF-1, or ICSBP, individually or in any combination of two (data not shown, n = 3). However, co-transfection with the single CYBB binding site construct and PU.1 with IRF-1 and ICSBP demonstrated a 3-fold increase in CAT activity relative to p-TATACAT (334 ± 20%, n = 3), consistent with an approximately linear contribution by each of the four CYBB sequences in the p-haf1TATACAT construct.

This functional interaction of PU.1 with PIP requires serine 148-phosphorylated PU.1 (41). To determine whether this PU.1/IP-1/ICSBP interaction with the CYBB promoter required serine phosphorylation, co-transfections were performed with PU.1 that had been mutated in various serine residues of the PEST domain (40), IRF-1, ICSBP, and p-TATACAT or p-haf1TATACAT. Only co-transfectants with the serine 148 mutant PU.1 had CAT activity that was significantly less than wild type PU.1 co-transfectants (Fig. 5C).

To determine whether the presence of the three factors, PU.1, IRF-1, and ICSBP, was sufficient to confer lineage-inappropriate expression from the −32 to −69 bp CYBB promoter sequence, p-TATACAT or p-haf1TATACAT was co-transfected into the epithelial cell line HeLa with expression vectors containing PU.1, IRF-1, and ICSBP. Absolute CAT activity of neither p-haf1TATACAT nor p-TATACAT was changed by co-transfection with PU.1 plus IRF-1 and ICSBP (Fig. 5C).

Several potential mechanisms have been implicated in lineage-specific gene regulation. In myeloid and B-cells, gene regulation appears to involve interaction between transcription factors of some lineage specificity, which confer greater lineage specificity by acting in combination. In these studies, we demonstrated that interaction between the ETS protein PU.1 and the IRF proteins ICSBP and IRF-1 was involved in lineage-specific expression of the CYBB gene, which encodes gp91phox.

This interaction required the presence of all three proteins simultaneously and increased gp91phox expression in myeloid cell lines.

Although the transcription factor PU.1 has been demonstrated to have a role in regulation of a number of myeloid genes (2, 6, 7), the present studies suggested a different mechanism for PU.1 than had been previously described. In regulation of some myeloid genes, PU.1 binding enhanced, but was not essential for, promoter function (2, 6). This was in contrast to the PU.1 binding elements in the CYBB gene and the NCF1 gene (encoding p47phox) (7). In both of these respiratory burst oxidase component genes, promoter mutation that abolished PU.1 binding also abolished promoter function. However, in contrast to functional studies of the NCF1 promoter element (7), our studies demonstrated that PU.1 alone was not adequate to confer lineage-inappropriate expression by the CYBB promoter element. Therefore, the NCF1 promoter element was functional in the absence of other lineage-specific factors in contrast to the requirement for IRF-1 and ICSBP with PU.1 for the CYBB promoter element.

B-cell-specific Igκ gene transcription required PU.1 to partner with the IFR protein PIP (8, 24), although ICSBP could substitute for PIP in activation of a reporter gene construct with the Igκ element (42). However, other functional partners for PU.1, with target genes for PU.1 partnering, had not previously been described. In these studies, we demonstrated that ICSBP and IRF-1 were functional partners for PU.1 interacting with the CYBB promoter element. This interaction required PU.1 serine 148, as had been described for PU.1-PIP partnering (8), suggesting a general pattern to PU.1 interaction with IRF proteins. However, in contrast to Igκ activation by PU.1-PIP, maximal activation of the CYBB promoter element required PU.1 interaction with both IRF-1 and ICSBP in a heterotrimer. This was a novel mechanism for PU.1 function. In the PU.1 interaction with PIP, the transcriptional activation domain of PU.1 was dispensable (41). Further investigations will determine if PU.1 participates in transcriptional activation of the CYBB gene directly or serves as an anchor for transcriptional activation by one or both of the IRF proteins.

It had been postulated that interaction between IRF-1 and ICSBP was an important mechanism for regulation of genes involved in the immune response (43). In one model of this interaction, ICSBP bound an ISRE or positive regulatory domain 1 element and functioned as a repressor (43). In cells treated with IFNγ, this repression was antagonized by IRF-1 binding to the same element and functioning as an activator (43). In co-transfection experiments with HeLa cells, other investigators demonstrated that overexpression of ICSBP repressed artificial promoter constructs containing various ISRE and positive regulatory domain 1 elements (44). However, in co-transfection experiments with the myelomonocytic cell line U937, these investigators also found that overexpression of ICSBP repressed some of these artificial promoter constructs but mildly activated others (44). These results suggested that the interaction of ICSBP with promoter elements might be more complex.

Recent work by another group of investigators provided additional insight into the complexity of interaction between
IRF-1 and ICSBP (12, 13). These investigators demonstrated that only nonphosphorylated ICSBP was capable of direct DNA binding and that ICSBP was phosphorylated constitutively in U937 cells (13). Phosphorylated ICSBP bound DNA indirectly via interaction with IRF-1, which bound DNA in response to IFNγ stimulation (13). This interaction required tyrosine phosphorylation of IRF-1 (13). These investigators, however, did not identify any target genes for this potential IRF-1 interaction with ICSBP. Our investigations identified a target gene for the IRF-1 interaction with ICSBP and described a functional interaction in which ICSBP is involved in activation, not repression of gene expression.

The current investigations suggested that the previously described HAF1 complex (20) was composed of PU.1 with either IRF-1 or ICSBP and that the newly described HAF1a complex was composed of PU.1 with both IRF-1 and ICSBP. According to this model, there were three possible interactions among PU.1, IRF-1, and ICSBP. In all of these interactions, PU.1 was postulated to function as the DNA-binding anchor due the inability of either IRF-1 or ICSBP alone, or IRF-1 together with ICSBP, to bind directly to the CYBB promoter sequence. Possible combinations consistent with the data were as follows: PU.1 bound IRF-1, which bound ICSBP; PU.1 bound ICSBP, which bound IRF-1; or PU.1 bound directly to both IRF-1 and ICSBP simultaneously. The third possibility seems to be the least likely due to the potential for steric hindrance and the known ability of the two IRF proteins to physically interact (12). Further investigations will clarify the interactions and determine the domains involved.

Overexpression of PU.1, IRF-1, and ICSBP in HeLa cells did not activate transcription from a co-transfected artificial promoter construct containing the HAF1/HAF1a binding CYBB promoter element. One possible explanation for this was that an additional, not yet identified, myeloid-specific transcription factor was involved. Another possible explanation was that the interaction of PU.1, IRF-1, and ICSBP required lineage-specific post-translational protein modification(s). According to this hypothesis, the essential protein modifications may have occurred when the proteins were overexpressed in the myeloid cell line, but not in the epithelial cell line.

Since serine 148 phosphorylation of PU.1 was necessary for functional interaction of PU.1 with IRF-1 and ICSBP, and tyrosine phosphorylation of IRF-1 and ICSBP was previously demonstrated to be necessary for interaction of IRF-1 and ICSBP with each other (13), it was possible that the mechanism of CYBB gene regulation required specific phosphorylation of the three proteins. Further, it was possible that this protein phosphorylation depended upon signal transduction pathways leading to kinase activity specific to myeloid cells. These possibilities are presently under investigation in our laboratory.

Regulation of gp91phox expression involves the coordinate action of a number of transcriptional repressors and activators (4, 29, 45). These factors have variable stringency of lineage restriction and are likely to confer lineage specificity by acting in combinations. Our investigations suggested that post-translational modifications, of variable lineage specificity, may also be involved. Further investigations of myeloid gene regulation will involve identification of the DNA-binding proteins, and also myeloid-specific signaling events that modify these proteins.