Multiple PU.1 sites cooperate in the regulation of $p40_{phox}$ transcription during granulocytic differentiation of myeloid cells

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The $p40_{phox}$ protein, a regulatory component of the phagocyte NADPH oxidase, is preferentially expressed in cells of myeloid lineage. We investigated transcriptional regulation of the $p40_{phox}$ gene in HL-60 myeloid cells. Deletion analysis of approximately 6 kb of the 5'-flanking sequence of the gene demonstrated that the proximal 106 base pair of the promoter exhibited maximum reporter activity. This region contains 3 potential binding sites consistent with the activity at multiple sites is required for PU.1-dependent expression was also observed in the nonmyeloid HeLa cell line, whereas the moderate level of promoter reporter activity in the nonmyeloid HeLa cell line was independent of PU.1. Chromatin immunoprecipitation assay demonstrated occupation of the PU.1 sites by PU.1 in vivo in HL-60 cells. Cotransfection of the pGL3-p40-106 reporter construct with a dominant-negative PU.1 mutant dramatically reduced promoter activity, whereas the overexpression of PU.1 increased promoter activity. Promoter activity and transcript levels of $p40_{phox}$ increased in HL-60 cells during dimethyl sulfoxide–induced differentiation toward the granulocyte phenotype, and this was associated with increased cellular levels of PU.1 protein. Our findings demonstrate that PU.1 binding at multiple sites is required for $p40_{phox}$ gene transcription in myeloid cells and that granulocytic differentiation is associated with the coordinated up-regulation of PU.1 and $p40_{phox}$ expression. (Blood. 2002;99:4578-4587)

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Introduction

NADPH oxidase is the major inducible source of superoxide and superoxide-derived reactive oxygen species in phagocytes and is a key enzyme in host defense against microbial infection. Genetic deficiency of this enzyme results in the inherited disorder chronic granulomatous disease (CGD), which is characterized by impaired phagocyte microbicidal activity and a clinical syndrome of recurrent life-threatening infections. The reactive oxygen species generated by this enzyme are also toxic to host cells and may account for much of the tissue injury that occurs during inflammation. Therefore, the expression and activity of the phagocyte NADPH oxidase components must be tightly regulated.

The fully assembled active NADPH oxidase complex is composed of membrane-bound and cytosolic components. The membrane-associated core component of the oxidase is a heterodimeric b-type cytochrome comprised of light ($p22_{phox}$) and heavy ($gp91_{phox}$) chain polypeptides. Cytosolic components include $p67_{phox}$, $p47_{phox}$, $p40_{phox}$, and the small GTPase protein, Rac2. X-linked CGD, the most prevalent form of CGD, is caused by mutations in the $gp91_{phox}$ gene, whereas autosomal recessive CGD is associated with mutations of the $p47_{phox}$, $p67_{phox}$, or $p22_{phox}$ genes.

Numerous studies have demonstrated essential roles for $gp91_{phox}$, $p22_{phox}$, $p67_{phox}$, $p47_{phox}$, and Rac2 in phagocyte NADPH oxidase function. Reconstitution of the oxidase by transfection into cell lines deficient in the oxidase proteins or recombination of the components in vitro shows that efficient superoxide generation by the NADPH oxidase complex is absolutely dependent on these components. The role of $p40_{phox}$, on the other hand, is less clearly defined because it does not appear to be absolutely necessary for the core enzymatic function, namely the univalent reduction of oxygen. Nevertheless, several lines of evidence suggest that $p40_{phox}$ is directly involved in the normal physiologic function of the oxidase. Thus, $p40_{phox}$ forms a trimolecular complex with $p47_{phox}$ and $p67_{phox}$ in the cytosol of resting phagocytes and on activation translocates to the membrane with these components. Moreover, $p40_{phox}$ can bind $p67_{phox}$ and $p47_{phox}$, and the strong binding of $p40_{phox}$ to $p67_{phox}$ can be disrupted by the activated guanosine triphosphate-bound form of Rac, a key intermediate in oxidase activation. The SH3 domain of $p40_{phox}$ competes for the C-terminal, proline-rich domain in the flavocytochrome. More recently, it has been reported that the 57-kd actin-binding protein coronin associates with the $p40_{phox}$–$p67_{phox}$–$p47_{phox}$ cytosolic complex through...
the C-terminal domain of p40phox, and that it colocalizes with the phox proteins in the periphery of the phagocytic vacuole during phagocytosis and activation of the oxidase.33 Finally, recent studies have shown that p40phox is hyperphosphorylated during NADPH oxidase activation and dephosphorylated during enzyme inactivation.34,35 In summary, these studies suggest that p40phox acts as a regulator of oxidase activity and as an adapter that links oxidase to the cytoskeletal elements that participate in cell activation.

Tissue distribution studies have demonstrated that, similar to the p91phox, p47phox, and p67phox components of NADPH oxidase, p40phox is preferentially expressed in cells of myeloid lineage.36,37 Transcription of many myeloid-species-specific genes is regulated by PU.1, a member of the ets family of transcription factors.38 Gene-targeting studies have demonstrated that PU.1 is essential for the normal embryonic development of hematopoietic cells of myeloid, B-cell, and T-cell lineages.39,40 We have previously characterized the promoter of the p40phox gene and demonstrated that PU.1 is essential for p40phox transcription in myeloid cells.41 PU.1 appears to regulate transcription by binding to a single cis element located in the proximal region of the p40phox gene. In this report, we describe our studies on the transcriptional regulation of the p40phox gene in the HL-60 myeloid cell line. Our data show that p40phox transcription is regulated by 3 PU.1 binding sites located within the proximal region of the p40phox promoter and that the PU.1 transcription factor binds to each site, in vitro and in vivo. The central role of PU.1 in the regulation of p40phox expression supports previous observations that this phox protein is mainly restricted to cells of myeloid lineage and is of particular importance in phagocyte function.

Materials and methods

Materials

RPMI 1640 was obtained from Life Technologies (Gaithersburg, MD). Restriction enzymes, T4 polynucleotide kinase, RNasin, and pGL3-Basic luciferase reporter vector and the dual luciferase assay kit were from Promega (Madison, WI). γ-[32P]Adenosine triphosphate (ATP), 6000 Ci/mmol (370 MBq/mol), was obtained from DuPont-NEN (Boston, MA). The TOPO-TA Cloning Kit, with the pCRll vector for cloning products of the polymerase chain reaction (PCR), was obtained from Invitrogen (San Diego, CA). Oligonucleotides were synthesized at the Advanced DNA Technology Unit (University of Texas Health Science Center at San Antonio). The Sequenase DNA sequencing kit was obtained from United States Biochemical (Cleveland, OH). PU.1 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Human p40phox genomic cloning and sequencing

The p40phox 5’-flanking region was cloned using the PromoterFinder Kit (Clontech, Palo Alto, CA) according to the manufacturer’s protocol. PCR was performed using the human genomic libraries provided as templates to amplify the desired sequences. The forward primer was complementary to the adaptor ligated to the genomic DNA fragments contained in each library. The reverse primer (5’-CCAGGGAGCAGGTGGAGAGTCTCGC-3’, Figure 2A) was complementary to base pair (bp) +142 to +117 of the p40phox gene.22,36 Amplified products were analyzed on a 1.2% agarose gel and then subjected to a second round of PCR with nested primers. The nested reverse primer (5’-AGGCTGAGTCCACCTCTCCTC-3’) was complementary to bp +105 to +81 of the gene. Final PCR products were then labeled with [32P]-dCTP and subjected to a second round of PCR with nested primers. The products were then labeled with [32P]-dCTP and subjected to agarose gel electrophoresis.

Luciferase vector construction

Reporter vectors were constructed in the pGL3-Basic luciferase vector. Promoter regions were amplified using human genomic DNA as template. The reverse primer was similar to the nested reverse primer used above, but a site for cleavage by XhoI was added (5’-CACCTGAGAGGTGAGTTCACTTCTC-3’). The forward primers corresponded to the upstream sequences of desired promoter regions, with a KpnI cleavage site at the 5’ end. PCR products were digested with KpnI and XhoI and were cloned into the pGL3-Basic reporter vector. Inserts of the constructs generated all extend downstream to +104 relative to the transcription start site of the p40phox gene.26 Mutations of the PU.1 binding sites were generated by site-directed mutagenesis (QuikChange kit; Stratagene, La Jolla, CA) using the primers illustrated in Figure 2B. Constructs were confirmed by restriction mapping and sequencing.

Cell culture and transient transfections

As previously described,41 the human myeloid cell lines HL-60, THP-1, U937, and PLB985 and the human B-cell line Raji were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 10 mM HEPES, penicillin, and streptomycin. Transfection was carried out by electroporation as we have described.41 Briefly, approximately 105 cells were resuspended in 0.5 mL complete medium containing 2 μg luciferase reporter constructs and 0.2 μg of a Renilla luciferase vector (pRL-null; Promega) as a transfection efficiency control. In Figure 1, transfection was also carried out with equimolar amounts (7 pmol) of each construct, plus an unrelated plasmid, pUC19, to provide an equal amount of total DNA. For cotransfection assays, 10 μg each reporter construct and PU.1 expression plasmid (a generous gift from Dr M. Klemsz, Indiana University, Indianapolis) were used. Electroporation was carried out at 960 μF and 250 V for HL-60 cells or 320 V for Raji cells. At 48 hours the cells were washed 3 times in phosphate-buffered saline (pH 7.4), lysed in 100 μL 1× reporter lysis buffer (Promega), and centrifuged at 400 g for 5 minutes at ambient temperature, and 20-μL aliquots of the supernatants were tested for reporter gene activity using the dual luciferase assay system (Promega) and a Turner Designs TD-20/20 luminometer. The human cervical carcinoma epithelial cell line HeLa was grown in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum and transfected with Lipofectamine Plus reagent (Gibco) according to the manufacturer’s protocol.

In vitro synthesis

Mouse PU.1 cDNA was subcloned into pBluescript SK and was transcribed in vitro using T3 RNA polymerase and the T7-coupled reticulocyte lysate system (Promega). Synthesized [35S]-methionine-labeled PU.1 was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography. A predominant band measuring approximately 38 kd was observed, consistent with the molecular mass previously reported.43 The control un-programmed sample (ie, no cDNA) produced no corresponding band.

Nuclear extracts

 Cells were disrupted by nitrogen cavitation using a technique originally described for neutrophils,44 and nuclear extracts were prepared as we have previously reported in detail.45 Extracts were collected and stored in aliquots at −70°C. The protein concentration was determined using the Bradford reagent (Bio-Rad, Hercules, CA).

Electrophoretic mobility shift assay

Complementary DNA oligonucleotides were annealed by heating in 1× NET at 95°C for 5 minutes and cooling at ambient temperature. Probes were then labeled with [γ-32P]ATP and T4 polynucleotide kinase. For gel shift assays, nuclear extract (6 μg) was incubated for 20 minutes at ambient temperature with 5 × 104 cpm of the labeled DNA probe in 20 μL binding buffer containing 10 mM Tris-HCl (pH 7.6), 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, 1 μg/μL bovine serum albumin, and 2 μg poly(dI-L-C). For supershift assays, 2 μL specific antibody was added, and
the reaction was continued for 15 minutes. Samples were loaded on 5% non-denaturing polyacrylamide gels, and electrophoresis was carried out at 200 V in 25 mM Tris (pH 8.5) with 190 mM glycerol and 1 mM EDTA. Competition assays were carried out in the same manner, except that the above reaction mixture was preincubated with competitor DNA for 10 minutes at 4°C before the labeled probe was added. Signals were quantitated with a PhosphorImager and ImageQuant software (Molecular Dynamics). Relative binding avidity of various DNA probes for PU.1 were determined by comparisons of band intensities observed in the presence of varied amounts of probes or a series of dilutions of competing oligonucleotides.

Chromatin immunoprecipitation analysis

Chromatin immunoprecipitation (ChIP) analysis was carried out as described by Boyd et al.45 Briefly, formaldehyde was added directly to the cell culture to a final concentration of 1% (wt/vol), and the cells were incubated at 32°C for 10 minutes. Glycine was added to stop the reaction, and the cells were collected, washed, and allowed to swell on ice for 10 minutes in 5 mM PIPES (pH 8.0) containing 85 mM KCl, 0.5% NP-40, 0.5 mM phenylmethylsulfonfluoride, and 100 ng/mL leupeptin and aprotonin. Pelleted nuclei were collected and incubated on ice for 10 minutes in 50 mM Tris HCl (pH 8.1), 1% SDS, 10 mM EDTA, and the protease inhibitors, sonicated on ice to break the chromatin DNA, and precleared with Staph A cells (protein A–positive Staphylococcus aureus cells; Roche Molecular Biochemicals, Indianapolis, IN). Precleared chromatin from 2 × 10⁷ cells was incubated with 1.5 μg affinity-purified rabbit polyclonal antibody to PU.1 (anti-PU.1 sc-352X; Santa Cruz Biotechnology) or without antibody, and it was rotated at 4°C for 12 to 16 hours. Immunoprecipitation was carried out with Staph A cells. The supernatant from the reaction lacking primary antibody was saved as total input of chromatin and was processed in the same way as the eluted immunoprecipitates, starting with the cross-link reversal step. Cross-linking was reversed by incubation at 65°C for 5 hours in the presence of 300 mM NaCl and 0.01% RNase A. Samples were then precipitated, resuspended, and treated with proteinase K, followed by extraction with phenol-chloroform-isoamyl alcohol and precipitation with sodium acetate and ethanol plus tRNA and glycogen as carriers. Pellets were collected, washed, and allowed to swell on ice for 10 minutes in 5 mM Tris HCl (pH 7.0), the membrane was stripped and probed with 32P-labeled cDNA for human acidic ribosomal phosphoprotein 36B4 as a loading control.

Northern blot analysis

Total cellular RNA was isolated using the Ultraspec reagent (Biotex Laboratories, Houston, TX) and dissolved in nuclease-free water. Samples of RNA (10 μg) were separated by electrophoresis in 1% agarose under denaturing conditions (formaldehyde), transferred to Nytran Plus membrane (Schleicher & Schuell, Keene, NH), UV cross-linked, and probed with p40phox cDNA labeled using the High Prime DNA labeling kit (Roche Molecular Biochemicals) and [α-32P]-cTP (DuPont-NEN). Following autoradiography at −70°C, the membrane was stripped and probed with 32P-labeled cDNA for human acidic ribosomal phosphoprotein 36B4 as a loading control.

Results

The 5′-flanking region of the human p40phox gene exhibits promoter activity in myeloid cells

Previous studies have shown that human p40phox mRNA is expressed almost exclusively in hematopoietic cells that exhibit NADPH oxidase activity.36,37 To identify the sites of promoter activity in the p40phox gene, regions of the 5′-flanking sequence were prepared by PCR, cloned into the luciferase reporter vector pGL3-Basic, and assayed by transient transfection and expression in the promyelocytic HL-60 cell line. The highest level of activity (approximately 37-fold increase over pGL3-Basic vector control) was observed with the construct pGL3-p40-106 (Figure 1), which contained the first 106 nucleotides upstream of the p40phox transcription start site.36 Constructs extending further upstream exhibited progressively lower luciferase activity, suggesting that the minimal p40phox promoter lies within the first 106 nucleotides of the 5′-flanking sequence of the p40phox gene and that negative regulatory elements may be present in upstream regions. Other myeloid cell lines tested (THP-1, U937, and PLB985) showed similar results (data not shown).

Three PU.1 consensus sites in the proximal p40phox promoter bind PU.1

Inspection of the p40phox promoter for potential regulatory elements identified several core-binding sequences (GAGGAA) for PU.1 transcription factor. Applying criteria for optimal flanking nucleotides that we identified previously,46 3 of these PU.1 sites (PU.1a, −95 to −100; PU.1b, −65 to −60; and PU.1c, +80 to +85; Figure 2A) were considered to have a high probability of binding PU.1. To determine whether these sites bound PU.1, electrophoretic mobility shift assay (EMSA) was carried out using 32P-labeled double-stranded oligonucleotides corresponding to these regions (Figure 2B) and nuclear extracts of HL-60 cells. Several DNA–protein complexes were formed between the PU.1 probes and HL-60 nuclear extracts (Figure 2C). Effective competition with excess unlabeled wild-type, but not mutated, DNA probes demonstrated that these bands were specific. The addition of specific antibody to PU.1 resulted in a dramatic decrease in band intensity and in the appearance of new supershifted bands, providing clear evidence that the complexes contained PU.1 protein. Comparison with previous studies suggested that the major bands of slower mobility contained intact PU.1 protein, whereas the faster-migrating bands were probably formed by PU.1 degradation products.46

Figure 1. Identification of the proximal promoter region of the p40phox gene. HL-60 cells in log phase of growth were transfected with the indicated constructs and assayed for luciferase activity after 48 hours. All p40phox constructs extended from the indicated position in the 5′-flanking sequence of the gene to nucleotide +104 of the 5′-UTR relative to the reported transcriptional start site.36 Luciferase activity is reported as the ratio of the test construct to the promoterless vector pGL3-Basic. Values were corrected for transfection efficiency by cotransfection with the renilla expression plasmid and were normalized to equal molar content of DNA. Data shown are means (± SE) of 5 independent experiments.
Intact PU.1 binding sites are required for promoter activity in HL-60 myeloid cells and Raji B cells, but not in nonmyeloid HeLa cells.

To investigate the role of these 3 p40phox PU.1 binding sites on promoter activity, a series of mutation and deletion promoter–luciferase constructs were prepared in which the 3 PU.1 sites were deleted or mutated individually or in combination. These constructs were tested as before by transient expression in HL-60 cells. Mutation of PU.1a, PU.1b, or PU.1c in the \( /H11002 \) 1599 bp p40phox promoter construct reduced activity by 82%, 63%, and 18%, respectively (Figure 3A). Similar results were observed with the deletion series (Figure 3B). The p40phox promoter activity in the \( /H11002 \) 1599 bp construct was nearly abolished in the HL-60 cells when all 3 binding sites were mutated, indicating the essential requirement for PU.1 in this cell type (Figure 3B). Because PU.1 and p40phox are also expressed in B lymphocytes, we investigated the roles of the PU.1 sites on p40phox promoter activity in this cell type using the Raji human B-cell line. The PU.1 sites in the p40phox promoter bound to PU.1 protein in Raji nuclear extracts (Figure 4A). Furthermore, the promoter was active in these cells, though at levels lower than those observed in the HL-60 cells (Figure 4B). More important, however, the promoter function was dependent on the presence of intact PU.1a and PU.1b sites (Figure 4B; PU.1c not tested). To investigate further the requirement for the PU.1 sites in p40phox promoter activity, the mutated constructs were also analyzed in the nonmyeloid HeLa cell line, which does not express PU.1. Although the activity of the wild type –1599 bp p40phox promoter construct was high in HeLa (Figure 4C), this activity was not affected by mutation of the PU.1 sites. The relatively high basal activity seen in HeLa cells with these constructs was surprising and might have been mediated by transcription factors present in these cells that bind the –1599 p40phox promoter construct at sites.

Figure 2. Characterization of 3 PU.1 binding sites in the p40phox promoter. (A) Sequence of the proximal promoter region of the p40phox gene. 3 PU.1 sites are underlined and labeled PU.1a, PU.1b, and PU.1c. The arrow indicates the reported transcription start site, and the translation initiation codon is double-underlined. Nested reverse primers used in cloning are in bold font. (B) Sequences of wild-type and mutated p40phox PU.1 oligonucleotides used in the EMSA studies. Core PU.1 binding domains are underlined, and mutated nucleotides are shown in bold. (C) EMSA of HL-60 nuclear extracts with \(^{32}P\)-labeled p40phox PU.1 DNA probes. HL-60 nuclear extract (5 \( \mu \)g) was incubated with the labeled probes alone (lanes 1, 5, and 9) or together with either antibody to PU.1 (lanes 2, 6, and 10) or a 200-fold molar excess of the homologous wild-type (lanes 3, 7, and 11) or mutated (lanes 4, 8, and 12) oligonucleotides (Figure 2B). The specific PU.1-DNA complex (PU.1) and the supershifted complex (SS) are indicated by arrows.

Figure 3. Effect of 3 PU.1 sites on p40phox promoter function in HL-60 cells. (A) Mutational analysis of the –1599 to +304 region of the p40phox promoter. The 3 PU.1 sites (open boxes) present in the pGL3-p40-1599 construct were mutated (hatched boxes) singly or in combination, and the resultant constructs were assayed for reporter gene activity in HL-60 cells as in Figure 1. The arrow indicates the reported transcription start site. (B) Deletion analysis of the contribution of each PU.1 site to the overall transactivation activity of the proximal promoter (–106 to +104 bp) of the p40phox gene. Deletion constructs were prepared and assayed for reporter gene activity in HL-60 cells as in Figure 1. Data shown in both panels are means (± SE) of 8 independent experiments. Analysis of variance showed that differences in luciferase activity among the constructs were significant \((P \leq .01)\), and t test demonstrated that the luciferase activities of the mutation and deletion constructs were significantly lower \((P \leq .01)\) except for the PU.1c mutation construct, where \(P = .044\) than those from wild-type counterparts.
distinct from the PU.1 elements. These sites might normally be repressed in nonmyeloid tissues in vivo through elements in the gene not included in the $p40^{phox}$ construct. Such factors would be absent, functionally inactive, or PU.1-dependent in HL-60 and Raji cells because in both cases, mutation of the PU.1 sites was sufficient to eliminate most promoter activity.

Promoter activity of the PU.1 sites correlates with PU.1 binding avidity

Our previous studies indicated a correlation between the transactivation activity of PU.1 sites from a number of promoters and their affinity for the PU.1 protein. Thus, we hypothesized that the different levels of transactivation activity of the PU.1 sites in the $p40^{phox}$ promoter might be attributed in part to their relative PU.1 binding avidity. To investigate this possibility, the binding affinities of the 3 sites for PU.1 present in HL-60 nuclear extracts or in vitro synthesized PU.1 were estimated by 2 methods. First, increasing amounts of each labeled $p40^{phox}$ PU.1 oligonucleotide probe were incubated with a fixed amount of PU.1 protein (Figure 5A). Second, the ability of unlabeled $p40^{phox}$ PU.1 oligonucleotide probes to compete for binding with the well-characterized $p47^{phox}$ PU.1 site was determined (Figure 5B). Semiquantitative analysis of these binding studies suggested that the binding avidity of the PU.1a site is 2- to 4-fold greater than that of the PU.1b site (compare lane 2 with lanes 8 and 9 in Figure 5A and lane 6 in Figure 5B). The binding avidity of PU.1b is, in turn, 3- to 8-fold greater than that of the PU.1c site (compare lane 7 with lane 15 in Figure 5A and lane 5 with lane 9 in Figure 5B).

Converting these EMSA results with the data obtained by deletion and mutation transfection studies indicated a direct relationship between the avidity with which these sites bind PU.1 and their capacity to dictate reporter gene transcription (PU.1a is greater than PU.1b is greater than PU.1c). However, the decrease in promoter activity on deletion of the PU.1c site was 20% when the other 2 PU.1 binding sites were intact, but it was 36% when the PU.1a site was deleted and 45% when the PU.1a and PU.1b sites were deleted (data not shown), suggesting that compensation may take place when one or another of the PU.1 sites is inactivated.

PU.1 binds to the $p40^{phox}$ promoter in vivo

To determine whether PU.1 protein binds the $p40^{phox}$ promoter at the identified PU.1 sites in vivo, we performed ChIP analysis on HL-60 cells using a specific polyclonal antibody to PU.1. Following immunoprecipitation and reversal of cross-linking, the DNA was purified and used as a template for PCR amplification using

Figure 4. PU.1 is present in nuclear extracts from HL-60 and Raji cells, binds to the $p40^{phox}$ promoter, and is required for reporter activity in Raji, but not HeLa cells. (A) EMSA of nuclear extracts with $^{32}$P-labeled $p40^{phox}$ PU.1 a DNA probe. HeLa (lanes 1-3), HL-60 (lanes 4-6) or Raji (lanes 7-12) nuclear extracts (5 μg) were incubated with the labeled probes alone (lanes 2, 5, and 8) or together with either antibody to PU.1 (lane 10) or a 200-fold molar excess of unlabeled PU.1a (lanes 3, 6, and 9), PU.1b (lane 11), or PU.1c (lane 12) oligonucleotide (see Figure 2B). The specific PU.1-DNA complex (PU.1) and the supershifted complex (SS) are indicated by arrows. Mutational analysis of the −1599 to +104 region of the $p40^{phox}$ promoter in Raji (B) or HeLa cells (C). The PU.1 sites (open boxes) present in the pGL3-p40-1599 construct were mutated (hatched boxes), and the resultant constructs were assayed for reporter gene activity as in Figure 1. Arrow indicates the reported transcription start site. Data shown are means (± SE) of 3 or more independent experiments.
primers that encompass the region of the p40phox promoter containing the 3 PU.1 binding sites. As a negative control, we carried out immunoprecipitation with antibody to c-Jun, consensus binding sites for which are absent from this region of the p40phox promoter. The p40phox primers produced an amplicon of predicted size from the total input DNA and the anti–PU.1-precipitated DNA, but not from anti–c-Jun–precipitated DNA (Figure 6). Furthermore, PCR primers for an unrelated gene, CCR5, generated products only from the total input DNA, confirming the specificity of the assay. The CCR5 primers were designed to amplify a region of the CCR5 gene that does not contain PU.1 or c-Jun binding sites. These data indicate that endogenous PU.1 protein in HL-60 cells binds the p40phox promoter in the region containing the PU.1 sites and thus may promote transcription of the gene in vivo.

Overexpression of PU.1 increases p40phox promoter activity, whereas a dominant-negative mutant of PU.1 decreases activity

To provide further evidence that PU.1 is critical for p40phox promoter activity, the p40phox luciferase construct pGL3-p40-106 or pGL-3-Basic control were transfected into HL-60 cells with or without cotransfection of either the PU.1 expression plasmid p6-mPU.1 or the dominant-negative mutant plasmid p66-NN, in which the transactivation domain is deleted. Overexpression of PU.1 increased the activity of the p40phox promoter by approximately 2.5-fold (Figure 7), whereas expression of the dominant-negative mutant PU.1 decreased activity by approximately 50%, presumably because of competition with endogenous PU.1. That the transactivation was dependent on the binding of PU.1 to the putative PU.1 sites in the proximal portion of the p40phox promoter was confirmed in control experiments using the pGL3-p40-106mt construct, in which all 3 PU.1 sites were mutated. No effect of wild-type or mutant PU.1 was observed with this mutant reporter construct (Figure 7).
Induction of granulocyte differentiation in HL-60 cells increases PU.1 protein, \( p40_{\text{phox}} \) mRNA expression, and \( p40_{\text{phox}} \) promoter activity

HL-60 cells can be induced to differentiate toward a granulocytic phenotype by treatment with dimethyl sulfoxide (DMSO).\(^{47}\) Components of the phagocyte NADPH oxidase system are induced by DMSO, and enzymatic activity can be detected within 1 to 2 days of treatment.\(^{48-50}\) Although \( p40_{\text{phox}} \) is not an essential component of the functional NADPH oxidase, it is expressed predominantly in phagocytic cells, complexed with the essential oxidase components \( p47_{\text{phox}} \) and \( p67_{\text{phox}} \), and, in all probability, has a role in enzyme activation and regulation.\(^{29,32}\) To investigate the influence of granulocytic differentiation on \( p40_{\text{phox}} \) gene expression, we first examined the induction of \( p40_{\text{phox}} \) mRNA during DMSO treatment. Total RNA was isolated from DMSO-treated HL-60 cells at daily intervals, and Northern blot analysis was carried out using a cDNA probe specific for human \( p40_{\text{phox}} \). Levels of \( p40_{\text{phox}} \) mRNA increased approximately 8-fold within 1 day of treatment (Figure 8A). Subsequently, transcript levels decreased but were maintained at approximately 4-fold over controls for 2 to 3 days. To determine whether increased gene transcription contributed to this observed increase in \( p40_{\text{phox}} \) gene expression, HL-60 cells were first transfected with the pGL-3–p40-106 reporter gene construct, then treated with DMSO for 38 hours, and luciferase reporter activity was measured. DMSO increased reporter gene activity by 3- to 4-fold over the untreated control (Figure 8B). However, when we transfected the construct in which all 3 PU.1 sites were mutated, only a small increase in luciferase activity was seen following DMSO treatment. This suggests that the increase in transcriptional activity induced by DMSO in the wild-type promoter was mediated through increased PU.1 activity. These data were supported by EMSA and immunoblot analysis because nuclear extracts from HL-60 cells taken 38 hours after DMSO treatment showed increased PU.1 binding activity (Figure 9A) and increased PU.1 protein using specific anti-PU.1 antibodies (Figure 9B).

**Discussion**

PU.1, the most divergent member of the \( ets \) family of transcription factors, is important in the early development of multiple hematopoietic progenitors. Targeted disruption of the PU.1 locus results in multilineage defects that affect B and T lymphocytes, monocytes, osteoclasts, alveolar macrophages, and neutrophils.\(^{39,40}\) The PU.1 transcription factor is expressed specifically in hematopoietic tissues, particularly in cells of granulocytic, monocytic, and B lymphoid lineages.\(^{42,51-53}\) These patterns of expression are shared with the components of the phagocyte NADPH oxidase.\(^{1-3}\) Moreover, previous studies have shown that the components of the phagocytic respiratory burst oxidase are transcriptionally regulated.
by PU.1. For example, expression of the large subunit of the membrane-bound cytochrome b558, gp91phox is controlled by PU.1, along with other transcription factors. Of particular note, the PU.1-binding site is critical for function of the promoter of the gp91phox (CYPB) gene, and point mutations in this site lead to the CGD clinical phenotype. In the case of the cytosolic oxidase component p40phox, we have shown that its myeloid-specific expression is regulated primarily through a single essential cis element that binds PU.1 with high avidity. Additionally, we and others have reported that p67phox transcription is regulated by a complex array of transcription factors that includes PU.1. Adding to the body of evidence for a key role of PU.1 in regulation of NADPH oxidase expression are our current data that the cytosolic oxidase component p40phox is also regulated by PU.1. We have shown that a unique combination of 3 PU.1-binding elements, 2 in the proximal promoter and one in the 5’ UTR of the gene, comprise the major regulatory components of p40phox gene transcription in the HL-60 myeloid and Raji B-cell lines. Moreover, we have demonstrated that the 3 PU.1-binding sites also mediate increased expression of the p40phox gene observed during DMSO-induced granulocytic differentiation of HL-60 cells.

Recent studies indicate that regulation by multiple PU.1 promoter elements may not be unusual for myeloid-restricted genes. Two functional PU.1 sites have been identified in the promoter of the myeloid-specific CD18 gene and, interestingly, in the promoter of the PU.1 gene itself. The PU.1-binding sites in the PU.1 promoter thus appear to act as a pathway for autoregulation, a potentially important concept for lineage development of myeloid cells. A recent report has indicated that a low level of expression of PU.1 in hematopoietic progenitors is associated with the development of B cells, whereas a high level of expression blocks B-cell development and promotes macrophage differentiation. Nevertheless, the specific functional implications of the presence of multiple functional PU.1 sites in a promoter are yet to be determined. It is possible that in some genes they could act in a synergistic or cooperative manner, allowing a relatively strong transcriptional response in the presence of low activity of the PU.1 transcription factor, a situation that might be found in the early stages of myeloid development. This may well be the case with the p40phox gene. Transcripts for the p40phox gene can be readily identified in undifferentiated myeloid cell lines by Northern blot analysis, whereas the mRNA for p40phox, p67phox, and gp91phox is generally more difficult to detect.

We showed previously that the avidity of binding of PU.1 by a particular cis element correlates with its capacity to dictate reporter gene transcription. The results of our analyses of the 3 PU.1 sites in the p40phox promoter support this earlier observation. The affinity of binding of PU.1 to the p40phox PU.1 oligonucleotide probes in the gel mobility shift assay and the transacting activity mediated by each site in the transfection studies were of the same rank order (PU.1a is greater than PU.1b is greater than PU.1c). The relatively high activity of the PU.1a site is probably caused by the presence of optimal (AT-rich) sequences flanking the 5’ end of the core GAGGAA sequence. Interestingly, these sequences are not found in the weakly acting PU.1c site. The proximity of the 3 PU.1-binding elements to each other and their different activities suggest that together they produce a synergistic activation of p40phox gene transcription. However, our deletion–mutation studies indicated that the relative contribution to promoter activity of each of the 3 PU.1 sites was not reduced when the other sites were functionally lost. Therefore, at least in resting HL-60 cells, it appears more likely that each PU.1 site acts independently of the others.

Transfection of the −1599 bp p40phox promoter construct into the nonmyeloid HeLa cell line resulted in moderate levels of reporter gene activity. Consistent with the absence of PU.1 protein in these cells, this activity was not affected by mutation of the PU.1 sites in the promoter construct. These data suggest that nonmyeloid-specific factors are capable of binding and transactivating the −1599 bp p40phox promoter construct in locations other than the PU.1 sites we have described here. However, if present in myeloid cells and B lymphocytes, these factors must be dependent on PU.1 binding because mutation of the PU.1 sites alone markedly reduced promoter activity in these cells. Given that the expression of p40phox is restricted to myeloid cells and B lymphocytes, transactivation of the gene by these putative factors in nonmyeloid tissues must be inhibited in some way. However, a previous report suggests that some myeloid-specific promoter–reporter constructs may show activity in HeLa cells, as opposed to other nonmyeloid cell types.

There is increasing evidence that PU.1 can act in association with accessory factors to transactivate genes. Early studies showed an essential functional requirement for the factor NF-EM5 (PPIP) in act in conjuction with PU.1 to transactivate the immunoglobulin kappa gene. The recruitment of NF-EM5 to the PU.1–DNA complex was dependent on the activation of PU.1 by phosphorylation of serine 148, possibly by casein kinase II. In the immunoglobulin kappa gene, nonphosphorylated PU.1 was able to bind its cognate site but could not stimulate promoter activity. It is possible that the binding of PU.1 to the p40phox gene recruits such accessory factors to a ternary complex that in turn dictates promoter activity. Our data do not directly support such a model because the pattern of complexes formed on EMSA with the p40phox PU.1 probes was similar for HL-60 nuclear extracts and in vitro–synthesized PU.1 protein. However, the recruitment of accessory factors may require additional nucleotide sequences that extend beyond those used for the probes.

The induction of granulocytic differentiation in HL-60 cells with DMSO led to an increase in the net cellular levels of p40phox mRNA. DMSO treatment also produced an increase in the total cellular levels of PU.1, particularly in the slower-migrating form of cellular levels of PU.1, which most likely corresponds to a hyper-phosphorylated form of the protein. These data are consistent with earlier studies using enriched human CD34+ progenitor cells in which the induction of myeloid differentiation with granulocyte macrophage colony-stimulating factor resulted in an increase in PU.1 mRNA and protein. The increase in PU.1 protein in the HL-60 cells was accompanied by an increase in the levels of PU.1 binding to DNA and PU.1-mediated p40phox promoter activity. Taken together with our functional mutation studies, these data suggest that the increased p40phox gene transcription observed during maturation and differentiation is mediated primarily by the 3 identified PU.1 sites. This does not preclude the contribution of additional accessory or coactivator factors, perhaps recruited through the activation and phosphorylation of PU.1. However, these putative factors would have to function through the PU.1 elements because mutation of these sites reduced the promoter activity in the HL-60 cells to near-background levels.

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Multiple PU.1 sites cooperate in the regulation of $p40_{phox}$ transcription during granulocytic differentiation of myeloid cells

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