Expression of the phagocyte cytosolic protein p47\textsubscript{phox}, a component of NADPH oxidase, is restricted mainly to myeloid cells. To study the cis-elements and trans-acting factors responsible for its gene expression, we have cloned and characterized the p47\textsubscript{phox} promoter. A predominant transcriptional start site was identified 21 nucleotides upstream of the translation initiation codon. To identify the gene promoter sequences, transient transfections of HL-60 human myeloid cells were performed with a series of 5'-deletion p47\textsubscript{phox}-luciferase reporter constructs that extended as far upstream as −3050 bp relative to the transcriptional start site. The −224 and −86 constructs had the strongest p47\textsubscript{phox} promoter activity, whereas the −46 construct showed a major reduction in activity and the −36 construct a complete loss of activity. DNase I footprint analysis identified a protected region from −37 to −53. This region containing a consensus PU.1 site bound specifically both PU.1 present in nuclear extracts from myeloid cells and PU.1 synthesized in vitro. Mutations of this site eliminated PU.1 binding and abolished the ability of the p47\textsubscript{phox} promoter to direct expression of the reporter gene. The p47\textsubscript{phox} promoter was active in all myeloid cell lines tested (HL-60, THP-1, U937, PLB-985), but not in non-myeloid cells (HeLa, HEK293). Finally, PU.1 transactivated the p47\textsubscript{phox}-luciferase constructs in HeLa cells. We conclude that, similar to certain other myeloid-specific genes, p47\textsubscript{phox} promoter activity in myeloid cells requires PU.1.

Polymorphonuclear neutrophils constitute the first line of host defense against many pathogenic bacteria and fungi. Their ability to kill invading microorganisms depends to a large extent on reactive oxygen intermediates generated by the phagocyte NADPH oxidase (1–3). The importance of the NADPH oxidase in the host defense system is emphasized by the genetic disorder chronic granulomatous disease, wherein victims suffer severe and recurrent infections because of a functionally defective NADPH oxidase (1, 2, 4–7). On the other hand, these highly toxic reactive oxygen species can cause significant tissue injury during inflammation (8–10). Thus, it is essential that their generation and the activity of the NADPH oxidase are tightly regulated.

The leukocyte superoxide-generating NADPH oxidase is a coordinated assemblage of the membrane-associated heterodimeric flavocytochrome b\textsubscript{558} (gp91\textsubscript{phox} plus p22\textsubscript{phox}) with four cytosolic factors, p67\textsubscript{phox}, p47\textsubscript{phox}, p40\textsubscript{phox}, and a small GTP-binding protein (Rac1/2) (7). As a general rule, the full complement of oxidase constituents is expressed only in phagocytic cells of the myeloid lineage, although exceptions have been noted. The gp91\textsubscript{phox} subunit of the cytochrome was found to be myeloid-specific, whereas p22\textsubscript{phox} is widely expressed, but not incorporated into membranes in the absence of the large subunit (11). Early studies on p47\textsubscript{phox} and p67\textsubscript{phox} showed expression restricted to neutrophils, other phagocytes, and B lymphocytes (12, 13), and similar myeloid specificity was subsequently reported for p40\textsubscript{phox} (14). Rac1 and Rac2, on the other hand, are widely expressed (15).

Systems or individual components similar to the phagocyte NADPH oxidase have been described in a number of other types of cells, including human fibroblasts (16–18), human glomerular mesangial cells (19, 20), rat glomerular epithelial cells (21), rat osteoclasts (22), and bovine (23) and human (24) endothelial cells. In at least some cases, these systems are not identical to the phagocyte NADPH oxidase. For example, the cytochrome b component in fibroblasts was reported to be structurally and genetically distinct from that in phagocytes (17). Moreover, the functional status of these non-phagocyte oxidase systems has not always been clear, as for instance in the case of human endothelial cells, which express the oxidase components at the transcript and protein levels, but lack the cytochrome b heme spectrum (24). In summary, current evidence indicates that p22\textsubscript{phox} and the Rac proteins are widely expressed, whereas the other oxidase components are expressed selectively, although not exclusively in myeloid phagocytic cells.

Transcriptional regulation of NADPH oxidase components has been described in most detail for the gp91\textsubscript{phox} subunit of cytochrome b (25, 26). The distal promoter region contains a CCAAT box motif that binds the transcription factor CP1. However, this interaction is prevented by a CCAAT displacement protein that binds to the region surrounding the CCAAT box, thereby repressing transcription. Down-regulation of the repressor appears to be required for expression of gp91\textsubscript{phox}.

Specific factors involved in the transcription of other components of NADPH oxidase have not been characterized. We have focused on the regulation of expression of p47\textsubscript{phox}, an essential cytosolic component of the phagocyte oxidase. In general, immature myeloid cells express little or no p47\textsubscript{phox}, whereas during differentiation transcripts and protein are induced in parallel with the acquisition of superoxide-generating activity (13, 27). The induction of p47\textsubscript{phox} gene expression occurs at the transcriptional level (13). Tumor necrosis factor, retinoic acid, 1,25(OH)\textsubscript{2}-vitamin D\textsubscript{3}, lipoteichoic acid, and lipopolysaccharide up-regulate p47\textsubscript{phox} gene expression (27–29). However, colony stimulating factor-1 has no effect (30), whereas interleukin-10 down-regulates expression (31). Interferon-γ decreases levels of p47\textsubscript{phox} mRNA and protein in mature phagocytes (32), but

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank\textsuperscript{TM}/EBI Data Bank with accession number(s) AF003533.

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enhances expression of p47phox induced by tumor necrosis factor, retinoic acid, and 1,25(OH)2-vitamin D3 in cultured myeloid cell lines (27, 28).

To understand better these complex events, we have initiated studies of transcriptional regulation of the p47phox gene by cloning, sequencing, and functionally characterizing its promoter. Here we report that the region of the first 86 base pairs of the p47phox gene 5′-flanking region possesses tissue-specific promoter activity in myeloid cells. The myeloid transcription factor PU.1 is absolutely required for this function.

**EXPERIMENTAL PROCEDURES**

**Materials**—RPMI 1640 was obtained from Life Technologies, Inc. and Serum Plus™ medium supplement from JRH Biosciences (Lenexa, KS). Restriction enzymes, T4 polynucleotide kinase, RNAsin, avian myeloblastosis virus reverse transcriptase, and pG3-L3 Basic luciferase vector and the luciferase assay kit were from Promega (Madison, WI). [γ-32P]ATP, 6000 Ci/mmol, was obtained from NEN Life Science Products. The TA Cloning Kit (containing the pCRII vector) for cloning products of the polymerase chain reaction (PCR) was obtained from Invitrogen (San Diego, CA). Oligonucleotides were synthesized by the Addgene DNA Technology Unit, University of Texas at Houston Medical Center. The Sequenase DNA sequencing kit was obtained from U. S. Biochemical Corp. PU.1 and PEA3 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

**Primer Extension Analysis—**Poly(A) RNA was isolated from HL-60 cells using oligo(dt)-cellulose (FastTrack Kit, Invitrogen). A reverse oligonucleotide primer corresponding to nt 43–19 of the cDNA (33) was labeled with [γ-32P]ATP and T4 polynucleotide kinase and 5 × 105 cpm co-precipitated with 2.5 μg of HL-60 poly(A) RNA and 15 μg of the total RNA. Primer-RNA hybridization was carried out overnight at 42 °C in 40 mM PIPES, pH 6.4, 1 mM EDTA, 400 mM NaCl, and 80% deionized formamide. Following precipitation and washing in ethanol, the primer-RNA complex was dissolved in H2O and reverse transcribed by incubating with 40 units of avian myeloblastosis virus reverse transcriptase at 42 °C for 90 min in a 25-μl reaction mixture containing 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 10 mM MgCl2, 0.5 mM spermidine, 10 mM dithiothreitol, 0.6 mM each of dATP, dGTP, dCTP, and dTTP, and 40 units of RNasin. The product was then digested with 40 ng of RNase A at 37 °C for 30 min, phenol/chloroform-extracted, precipitated in ethanol, and separated on a sequencing gel. On the same gel a standard dyeoxy sequencing reaction using the same oligonucleotide primer and the plasmid containing ~1.2 kb of p47phox genomic sequence as template was also separated for identification of the bases corresponding to the transcription start site (TSS). Autoradiography using Kodak X-OMat film was carried out at ~70 °C.

**Human p47phox Genomic Cloning and Sequencing—**The p47phox 5′-flanking region was cloned by using the PromoterFinder Kit (CLON-TECH, 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 in each library. The reverse primer (5′-CTGGGTACCGAAGGCCTCTCTCAAGC-3′, see Fig. 1) corresponded to bp 75–50 of the p47phox cDNA. The amplified products were analyzed on a 1.2% agarose gel then subjected to the second PCR with nested primers. The reverse primer (5′-GGGCGATGTGACGAGTAAGGTGC-3′) was used to confirm the sequence of the cDNA.

**Luciferase Vector Construction—**Reporter vectors were constructed in the pG3-L3 Basic luciferase vector. The promoter regions were digested with XhoI and HindIII from the pCRII-p47phox clones were subcloned into promoterless luciferase reporter plasmid pG3L3-Basic at the same restriction sites. The constructs generated, the inserts of which range from downstream to +52 relative to the TSS of the p47phox gene, use the p47phox translation initiation codon ligated in-frame to the luciferase open reading frame.

For deletion construction PCR was done using the plasmid construct pG3L3-p47phox-1217 (i.e. from –1217 relative to the TSS, downstream to +52 followed by the luciferase open reading frame) as template, a luciferase antisense oligonucleotide (pG3-primer2, CTTCATGTTTTTC-GCCTCTCC) as the reverse primer and oligonucleotides synthesized with T4 polynucleotide kinase restriction site linked to the desired 5′-terminus of the p47phox promoter as the forward primers. The PCR-amplified products were digested with XhoI and HindIII and ligated to Xho/HindIII-digested pG3-L3 Basic.

For PU.1 binding site mutation, we used a site-directed mutagenesis kit (QuikChange, Stratagene, La Jolla, CA). The mutagenic primer (with altered nucleotides underlined) was 5′-CAAAAGCGACTTGGTTCTTTTAGGTGTTTGAGTTC-3′. The constructs were confirmed by restriction mapping and sequencing.

**Cell Culture—**The human promyelocytic cell line HL-60 was grown in RPMI 1640 medium supplemented with 10% Serum Plus and 10 mM HEPES. For 1 day prior to transfection and after transfection, the cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum. The human monocytic cell line THP-1, the promonocyte cell line U937, and the myeloid leukemia cell line PLB-985 were maintained in RPMI 1640 supplemented with 10% fetal bovine serum. Two non-myeloid cell lines, the human cervical carcinoma epithelial cell line HeLa and the transformed human embryonic kidney cell HEK293, were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. All media contained penicillin and streptomycin.

**Transient Transfections—**The cells were maintained at a density of about 5 × 105 cells/ml. Transfection was carried out by electroporation. Briefly, cells were resuspended in medium containing 20 μg of the luciferase reporter constructs and 4 μg of a cytomegalovirus-β-galactosidase vector (pCMV-β-gal) as a transfection efficiency control. Electroporation was accomplished at 960 microfarads and 250 V. At 48 h the cells were washed three times in phosphate-buffered saline, pH 7.4, lysed in 100 μl of 1 × reporter lysis buffer (Promega), and centrifuged at 12,000 rpm at ambient temperature, and 20-μl aliquots of the supernatants were tested in the luciferase assay system (Promega) using a Turner TD-20e luminometer. β-Galactosidase was assayed using a microassay procedure (34) and standardized with purified β-galactosidase (Sigma).

**In Vitro Translations—**The mouse PU.1 cDNA (a gift of Dr. M. Klemas, Indiana University, Indianapolis, IN) was excised by digestion with EcoRI and then inserted into pBluescript SK. A clone with the desired orientation was transcribed and translated in vitro using T3 RNA polymerase and the Tnt-coupled reticulocyte lysate system (Promega). The synthesized [35S]methionine-PU.1 was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. A predominant band of approximately 38 kDa was observed, consistent with the molecular mass previously reported (35). The control un-programmed sample (i.e. no cDNA) gave no corresponding band.

**Nuclear Extracts—**HL-60 cells were disrupted by cavitation using a technique described previously for polymorphonuclear neutrophils (36). The cells were washed twice in phosphate-buffered saline, pH 7.4, resuspended in 10 ml of cold relaxation buffer (100 mM KCl, 3 mM NaCl, 3.5 mM MgCl2, 10 mM PIPES, pH 7.3), and 3.5 μl of diisopropyl fluorophosphate (Sigma) were added. The cells were kept on ice for 10 min, then centrifuged at 400 × g for 5 min. The cell pellet was resuspended in 10 ml of relaxation buffer and pressurized in N2 at 350 p.s.i. for 20 min in a nitrogen bomb (Parr Instrument Co., Moline, IL) and released into 750 μl of a solution containing 20 mM EDTA, 100 mM MgCl2, 20 mM DTT, 4 mM phenylmethylsulfonyl fluoride, and 2 mM sodium orthovanadate. The cavitated cells were centrifuged at 400 × g for 10 min at 4 °C, the nucleus-enriched pellet resuspended and further purified on a discontinuous gradient of sucrose (0.5 to 0.88 M). The nuclear fraction was extracted in ~100 μl of urea extraction buffer (1.1 mM urea, 1% Nonidet P-40, 5% glycerol, 0.5 mM MgCl2, 5 mM KCl, 0.05 mM EDTA, 5 mM HEPES, pH 7.9) and microcentrifuged. The supernatant was collected and stored in aliquots at ~70 °C. The protein concentration was determined using the Bradford reagent (Bio-Rad).

**DNase I Protection Assay—**Plasmid constructs were linearized with MluI and end-labeled with [α-32P]dCTP and Klenow DNA polymerase. The insert was then excised by digestion with HindIII and gel-purified. The nuclear extract was precipitated for 30 min on ice, mixed with 50 mM of buffer containing 50 mM NaCl, 0.1 mM EDTA, 0.5 mM DTT, 10% glycerol, 20 mM HEPES/KOH, pH 7.5, 2 μg poly-dIIC). The labeled DNA (~100,000 cpm) was then added, and the incubation was continued at 25 °C. After 15 min, 5 μl each of 50 mM MgCl2, 10 mM CaCl2, and diluted DNase I were added. The incubation was continued for 1 min prior to the addition of 100 μl of stop solution (0.375% SDS, 15 mM EDTA, 100 mM NaCl, 100 mM Tris-HCl, pH 7.6). Calf thymus DNA (10 μg) and pro-
teinase K (20 μg) were added and the mixture incubated at 37 °C for 15 min, then 95 °C for 5 min, and phenol/chloroform-extracted and ethanol-precipitated. Finally, the DNA was separated on 6% denaturing polyacrylamide gels, using Maxam-Gilbert G1A sequencing reactions of the labeled fragments as the markers.

**Electrophoretic Mobility Shift Assay (EMSA)—** Complementary DNA oligonucleotides were annealed by heating in 1× NETr at 95 °C for 5 min 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 min at ambient temperature with 5 × 10^4 cpm of the labeled DNA probe in 20 μl of binding buffer containing 10 mM Tris-HCl, pH 7.6, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, 1 μg/ml bovine serum albumin, 2 μg/ml poly-d(I-C). For supershift assays, 2 μg of specific antibody was added and the reaction continued for 15 min. Samples were loaded on 6% nondenaturing 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 min at 4 °C before addition of the labeled probe.

**RESULTS**

Cloning of the p47phox 5′-Promoter Region—The p47phox promoter region was cloned using the PromoterFinder kit (CLONTECH). The nested reverse primers for the first and second round PCR (see Fig. 1) were derived from the p47phox cDNA to obtain the specific products. The PCR products amplified from 4 of 5 human sub-genomic libraries appeared as single bands, each of a different size (3.0, 2.2, 1.4, and 1.2 kb), on agarose gels. These products were cloned into the pCRII vector and their identification verified by direct sequence continuity of the 3′ ends with the 5′ portion of p47phox cDNA. Fig. 1 shows the complete sequence of the longest clone. This 5′-flanking region, like the promoters of other myeloid-specific genes such as CD11b and CD18 (37, 38), lacks a proximal authentic CAAT or TATA box, but the sequence TTTAA at bp −228 relative to the TSS (see below), flanked on both sides by G1C-rich sequences, has some similarity to the most common consensus sequence TATAAAA. Moreover, the initiator sequence, which appears to direct the site of initiation and basal level of transcription in many TATA-less promoters (39), was not present. However, sequence motifs were found in the p47phox promoter that correspond to binding sites of a number of known transcription factors. These include several purine-rich elements of the type that bind to the ets family of transcription factors. One of them, located at bp −45, is identical to the binding site for PU.1, a factor required for terminal myeloid gene expression and dif-
shown). This location maps to within one nucleotide of a pre-
lation initiation codon. Negative control reactions (HL-60 RNA replaced
by yeast tRNA) produced no bands (data not shown).

Localization of p47<sub>phox</sub> Promoter Activity—To identify cis-
elements and trans-acting factors important in p47<sub>phox</sub> promotor activity, we linked 8 cloned fragments of the p47<sub>phox</sub>
genomic DNA ranging in size from 3050 to 36 bp to the lucif-
erase reporter gene. These constructs and the promoterless vector pGL3-Basic were co-transfected into HL-60 cells with pCMV-β-gal (to correct for differences in transfection efficiency). Fig. 3 shows the normalized data. An increase of ~30-fold in luciferase activity was observed in lysates prepared from cells transfected with pGL3-p47–224 and pGL3-p47–86 constructs (the first 224 or 86 nucleotides upstream from the TSS, respectively) compared with pGL3-Basic vector. The shorter construct pGL3-p47–46 was less than 30% as active, while the shortest one pGL3-p47–36 was completely inactive, indicating that sequences critical for p47<sub>phox</sub> promoter activity are located between bp −86 and −36. Constructs extending further 5’ gave gradually less luciferase activity, suggesting the presence of negative regulatory elements upstream from bp −224 of the p47<sub>phox</sub> promoter.

A Protected Region in the Proximal Promoter Is a PU.1 Consensus Site—To test for protein-binding sites in the proximal 5’-flanking region required for myeloid promoter activity of the p47<sub>phox</sub> gene, we used DNase I footprinting. The p47<sub>phox</sub> genomic DNA fragment extending from bp −86 to +52 was analyzed using a nuclear extract from HL-60 cells. As illustrated in Fig. 4, increasing amounts of HL-60 nuclear extract showed graded protection from DNase I digestion of a 16-nt region between nt −37 and −52 (5’-AAAGAGGAATCGCTT-3’; lower strand), compared with the probe in the absence of nuclear extract. The protected sequence corresponds exactly to the PU.1 consensus motif GAGGAA (47).

The Potential PU.1 Binding Site Is Bound Specifically by PU.1 from Myeloid Cells and in Vitro Synthesized PU.1—To provide specific evidence that the p47<sub>phox</sub> promoter-binding activity was PU.1, we performed the EMSA (Fig. 5A). Double-stranded oligonucleotide encompassing the protected region of the p47<sub>phox</sub> promoter was bound by in vitro-translated PU.1 (lanes 4 and 8), but not by unprogrammed reticulocyte lysate (lane 3). Mutation of the PU.1 site on the lower strand from

FIG. 2. Mapping of the p47<sub>phox</sub> gene transcriptional start site by primer extension. An antisense oligonucleotide complementary to bp 43–19 of the cDNA was end-labeled and hybridized to RNA of MeSO-treated HL-60 cells. The primer was extended by reverse transcription, and the product was analyzed by gel electrophoresis alongside a set of Sanger sequencing reactions (lanes G, A, T, and C as marked) primed with the identical oligonucleotide. An arrow indicates the extended product, which corresponds to 21 nt upstream from the transcription initiation codon. Negative control reactions (HL-60 RNA replaced by yeast tRNA) produced no bands (data not shown).

FIG. 3. Characterization of the functional promoter of the p47<sub>phox</sub> gene. Log phase HL-60 cell cultures were transfected with the indicated constructs. All p47<sub>phox</sub> constructs extended from the indicated residue of the promoter (i.e. −3050, −2151, −1392, −1217, −224, −86, −46, or −36) downstream to residue +52. Luciferase activity was determined 48 h post-transfection and reported relative to the base-line activity of the promoterless construct (pGL3-Basic). Values were corrected for transcription efficiency by co-transfection of a β-galactosidase expression plasmid. Data (mean ± S.E.) shown are from at least five independent experiments.

p47<sub>phox</sub> Promoter Activity of PU.1
GAGGAA to CACCAA (48) eliminated this binding (lane 7). Nuclear extract from HL-60 cells bound to the 32P-labeled wild type oligonucleotide probe (Fig. 5, panel A, lane 9; panel B, lane 2) and gave a major shifted band that had an identical electrophoretic mobility to that observed with in vitro translated PU.1 protein. In the nuclear extracts, several complexes migrating faster and slower than in vitro translated PU.1 protein are also observed, both of which have been reported previously (48–50). The faster represent DNA-binding complexes formed by proteolytic products of PU.1, whose PEST domain renders it susceptible to protease cleavage. The slower ones may be due to the association with other transcription factors. Abrogation of all of these species by 250- or 50-fold molar excess of the unlabeled wild type probe (Fig. 5B, lane 4 or 5, respectively), but not by the probe with the mutated PU.1 binding site (Fig. 5B, lane 6), demonstrates binding specificity.

We next confirmed that the HL-60 cell-derived binding species that comigrated with in vitro translated PU.1 indeed represented PU.1. Incubation of the in vitro translated PU.1 with PU.1-specific antibody (against the epitope corresponding to amino acids 251–271 mapping to its carboxyl terminus) inhibited this binding species and resulted in supershifted complexes (Fig. 5A, lane 5). Addition of the PU.1 antibody to the reaction of the oligonucleotide probe and HL-60 nuclear extract also inhibited binding and generated similar supershifted complexes (Fig. 5B, lane 3), whereas addition of antibody to PEA3 (another member of the Ets family) had no effect (not shown).

**PU.1 Site Mutations Abolish p47phox Promoter Activity**—To verify the importance of the PU.1 binding site for myeloid-specific p47phox expression, we mutated this site (48) and tested for effects on p47phox promoter function. As shown in Fig. 5, an oligonucleotide with a mutated PU.1 site did not compete with PU.1 binding to the wild type region and did not bind to in vitro translated PU.1 or PU.1 from HL-60 nuclear extracts. This same mutation was then introduced by PCR into three of the p47phox promoter-luciferase constructs to form pGL-p47–2151Mt, pGL3-p47–224Mt, and pGL3-p47–86Mt. Transient transfection experiments comparing the wild type plasmid constructs and their mutated counterparts demonstrated that mutation of the PU.1 binding site reduced promoter activity to the control promoterless level (Fig. 6), implying that PU.1 binding is essential for p47phox promoter activity in HL-60 cells.

The p47phox Promoter Is Myeloid Tissue-specific—The documentation in the literature that expression of both PU.1 and
p47<sub>phox</sub> is selective for myeloid cells and B lymphocytes in vivo is consistent with our observation that PU.1 is essential for p47<sub>phox</sub> promoter activity. To verify this in our experimental system, we transfected the pGL3-p47–86 construct into myeloid (HL-60, THP-1, PLB-985, and U937) and non-myeloid (HeLa, HEK293) cell lines. As shown in Fig. 7A, the pGL3-p47–86 construct is active in all the myeloid cell lines tested, exhibiting a 15- to 36-fold increase in reporter activity, while the activity in non-myeloid cells is negligible, indicating that tissue specificity of the p47<sub>phox</sub> promoter is retained, even in the first 86 bp of the 5 '-flanking region.

We reasoned that if the myeloid tissue specificity of p47<sub>phox</sub> gene expression is due to PU.1 binding specifically to the promoter, PU.1 binding activity should be detected in these cells by EMSA. As shown in Fig. 7B, strong PU.1 binding activity was observed in the nuclear extracts of myeloid cell lines HL-60 and THP-1, but was undetectable in the non-myeloid HeLa cell extract.

**PU.1 Trans-activates the p47<sub>phox</sub> Promoter**—To demonstrate that PU.1 is critical for p47<sub>phox</sub> promoter activity, co-transactivation experiments were performed with the non-myeloid HeLa cell line, which lacks endogenous PU.1. The promoterless luciferase expression vector pGL3-Basic or the p47<sub>phox</sub> luciferase construct pGL3-p47–46 or pGL3-p47–86 was transfected into HeLa cells with and without co-transfection of the PU.1 expression plasmid PJ6-mPU.1 (Fig. 8). Co-transfection of PU.1 increased the activity of the p47<sub>phox</sub> promoters about 3-fold. That the trans-activation depended on binding of PU.1 to the putative site in the proximal portion of the p47<sub>phox</sub> promoter was confirmed by transfecting the PU.1 binding site-mutated p47<sub>phox</sub> luciferase construct PGL3-p47–86Mt together with the PU.1 expression vector. No increase in reporter gene expression was observed with the mutated construct.

**DISCUSSION**

Our results demonstrate that PU.1 plays an essential role in basal transcription of the p47<sub>phox</sub> gene. In transient transfection studies, the pGL3-p47–86 and pGL3-p47–46 constructs, both containing the PU.1 binding site, showed promoter activity in myeloid cells at full or reduced levels, respectively, whereas the PU.1 site-deleted construct PGL3-p47–36 lost promoter activity completely. Co-transfection of a PU.1 expression vector resulted in trans-activation of the p47-luciferase reporter constructs. Footprint analysis revealed a DNase I-protected site from −37 to −52. The protected region containing a consensus PU.1 site bound specifically to PU.1 extracted from myeloid cells and to that synthesized in vitro. Importantly, mutation of the PU.1 site in constructs PGL3-p47–86Mt, PGL3-p47–224Mt, and PGL3-p47–2151Mt abolished promoter activity completely, highlighting the essential role of the PU.1 element in p47<sub>phox</sub> promoter function. This feature contrasts with many other myeloid-specific genes that exhibit only partial dependence on PU.1 binding activity (51, 52). In the case of CD11b gene expression Sp1 binding is essential, although PU.1 also plays an important role (42). The mechanism by which PU.1 operates to contribute to p47<sub>phox</sub> gene transcription is unclear, although it is attractive to hypothesize that during assembly of the transcription initiation complex, PU.1 promotes the interaction between Sp1 and the TFIID complex by recruiting TATA-binding protein (TBP) (53, 54). However, using TBP-specific antibody we did not detect TBP association with the PU.1-DNA complex by EMSA (data not shown).

The PU.1 proto-oncogene was first identified 10 kb downstream of the site of Friend erythroleukemia virus integration in virally induced tumors. Viral integration results in overex-
expression of PU.1 mRNA in erythroid cells, an event linked to tumorigenesis (55). PU.1 is expressed exclusively in the hematopoietic system at high levels in B lymphocytes, granulocytes, and monocytic cells and at lower levels in immature erythroid cells (40). Since p47 phosphorylase is expressed selectively in myeloid cells and B lymphocytes (12, 13), it shares with PU.1 similar tissue specificity. PU.1 gene knockout mice show a multilineage defect in development of B and T lymphocytes, monocytes, and granulocytes (56). An in vitro system based on differentiation of embryonic stem cells into hematopoietic cells revealed that early myelopoiesis is relatively unaffected by a mutation in the PU.1 gene, but later developmental events are blocked and differentiation markers, CD-11b, M-CSFR, and CD-64, are absent (57).

The PU.1 consensus binding sequence GAGGAA is present in several myeloid-specific promoters and has been shown to be critical for the expression of CD11b and CD18 (49, 58). The PU.1 binding site in the p47 phosphorylase promoter matches at 12 of 14 nt with the SV40 PU.1 binding site (59), the first defined and best known example. Compared with previously reported PU.1 binding sites, the p47 phosphorylase site is one of the best matches to the SV40 site. However, the critical nucleotides reside at the central consensus sequence since mutations of this sequence eliminate p47 phosphorylase promoter activity and the binding to PU.1 protein. Of note, in the CD11b promoter PU.1 binds to a site that does not conform to the consensus sequence, yet an upstream GAGGAA sequence is not bound by PU.1 (58). Furthermore, the PU.1 requirement for DNA binding is confined to a short core sequence that has a high statistical representation in eukaryotic genomes. Thus, the GAGGAA sequence is not guaranteed to be a sequence that has a high statistical representation in eukaryotic genomes (63). Interestingly, this motif is a target for binding of Sp1 in the genes for human medium chain acyl-CoA dehydrogenase (64) and murine 1,4-galactosyltransferase (65), but not human phenylalanine hydroxylase (66). Moreover, the CTC box appears to be unique to the promoters of extracellular matrix proteins, binding a novel protein or protein complex in these genes (67). Whether this promoter region of p47 phosphorylase binds to myeloid-specific or more broadly acting transcriptional factors remains to be elucidated.

Recently, Gorlach et al. (68) reported a p47 phosphorylase pseudogene that is highly homologous to the gene itself, with the notable exception of a GT deletion at the beginning of exon 2 leading to a frameshift and premature stop codon. These authors suggested that recombination between the wild type p47 phosphorylase gene and its pseudogene is the main cause of the autosomal recessive form of chronic granulomatous disease associated with absence of p47 phosphorylase protein. The intron-exon structures of the wild type and pseudogene are identical, they are similarly transcribed and there are no major differences in their proximal promoter regions (68). Our new data are compatible with this observation. We found only minor sequence differences in the promoter regions among the four original constructs pGL3-p47-3050, pGL3-p47-2151, pGL3-p47-1392, and pGL3-p47-1217, generated from each of four sub-genomic libraries. There are minor variations between our clones and the sequence in the GenBank™ data base (accession number U33006) submitted by Thrasher. Nevertheless, all of these clones contain the PU.1 binding site at -45, and the sequences from bp -86 to -46 are identical among the clones, indicating the validity of the conclusion of this study that PU.1 is essential for p47 phosphorylase promoter activity in myeloid cells.

The HL-60 cell line was derived from a patient with promyelocytic leukemia (69). MeSO treatment induces its differentiation along the neutrophilic lineage, and p47 phosphorylase expression increases dramatically due to the induction of transcription of this gene (12, 13). We predicted that the p47 phosphorylase-luciferase reporter constructs used in the current work would express greater promoter activity in MeSO-differentiated HL-60 cells than in undifferentiated HL-60 cells. In preliminary experiments this does not appear to be the case (data not shown). Two potential explanations come to mind. First, the MeSO response elements of the p47 phosphorylase gene may reside outside of our p47 phosphorylase-luciferase constructs, including the longest one pGL3-p47-3050, either further upstream or downstream in an intron. Second, in addition to the control of initiation of gene transcription, elongation blockade, usually soon after initiation, is emerging as an important mechanism in regulation of gene expression (70). The increase in p47 phosphorylase mRNA during MeSO-induced differentiation could be due to a release of elongation blockade imposed after transcription initiation in undifferentiated HL-60 cells. We are currently testing these interesting possibilities.

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