Transcriptional Regulation of the p67phox Gene
ROLE OF AP-1 IN CONCERT WITH MYELOID-SPECIFIC TRANSCRIPTION FACTORS*

Received for publication, July 1, 2001
Published, JBC Papers in Press, August 1, 2001, DOI 10.1074/jbc.M106111200

Sen-Lin Li, Anthony J. Valente, Long Wang, Maria J. Gamez, and Robert A. Clark‡
From the Department of Medicine, University of Texas Health Science Center and South Texas Veterans Health Care System, Audie L. Murphy Division, San Antonio, Texas 78229-3900

We have investigated the myeloid-specific transcriptional regulation of p67phox, an essential component of phagocyte respiratory burst NADPH oxidase. Analysis was carried out on the p67phox 5′-flanking region from –3669 to –4 (relative to ATG), including the first exon and intron and part of the second exon. The construct extending from –985 to –4 produced the highest luciferase activity in myeloid HL-60 cells but was not active in HeLa or Jurkat cells, indicating myeloid-specific expression. Four active elements were identified: Sp1/Sp3 at –694, PU.1 at –289, AP-1 at –210, and PU.1/HAF1 at –182, the latter three being in the first intron. These cis elements bound their cognate transacting factors both in vitro and in vivo. Mutation of the Sp1, PU.1, or PU.1/HAF1 site each decreased promoter activity by 35–50%. Mutations in all three sites reduced promoter activity by 90%. However, mutation of the AP-1 site alone nearly abolished promoter activity. The AP-1 site bound Jun and Fos proteins from HL-60 cell nuclear extract. Co-expression with Jun B in AP-1-deficient cells increased promoter activity by 3-fold. These data show that full p67phox promoter activity requires cooperation between myeloid-specific and nonmyeloid transcription factors, with AP-1 being the most critical for function.

The NADPH oxidase of phagocytic cells is a key component of the host antimicrobial defense system. The oxidase is composed of the membrane-associated heterodimeric flavocytochrome b558 (gp91phox and p22phox) and the cytosolic factors p47phox, p67phox, p40phox, and Rac1 or Rac2 (1, 2). Following stimulation of resting phagocytes, the cytosolic factors translocate to the membrane to form a complex with the flavocytochrome, which generates superoxide by the transfer of electrons from NADPH to molecular oxygen. The reactive oxygen species derived from superoxide are mediators of microbial death, but they can also cause injury to host tissues during the inflammatory process.

Expression of the gp91phox, p67phox, p47phox, and p40phox components of NADPH oxidase shows tissue selectivity for cells of myeloid lineage, such as neutrophils, eosinophils, and monocyte/macrophages (3–5). In contrast, the Rac proteins and p22phox show a much broader distribution (6). Cellular models of granulocyte differentiation indicate that gp91phox, p67phox, and p47phox are not expressed in immature myeloid precursor cells but are induced during the differentiation/maturation process (4, 7–10). The p40phox protein, on the other hand, can be detected in undifferentiated myeloid cell lines, and its expression is increased during MeSO4-induced granulocyte differentiation (10–12). Thus, the phox proteins are coordinately up-regulated during terminal differentiation and maturation, suggesting shared mechanisms in the control of their expression. However, variations in the kinetics of induction indicate that regulatory factors unique to each gene may also be important.

Major contributions to the regulation of expression of the components of the phagocyte NADPH oxidase occur at the level of transcription. Studies of the gp91phox promoter have demonstrated a complex regulatory mechanism involving both positive and negative transcription factors (13–16). The upstream promoter contains a CCAAT-box motif that binds the transcription factor CP1, ISRE sequences that can bind IRF-1/2, and binding sites for a novel transacting factor termed BID. It is thought that in undifferentiated myeloid precursor cells, the positive promoter effect of these elements is masked by CDP, a CCAAT-displacement protein that binds the regions around these motifs, thereby suppressing transcription (13, 15). Down-regulation of the repressor protein during terminal differentiation is required for the expression of gp91phox. A critical regulatory element in the transactivation of gp91phox is a consensus sequence for the ets family of transcription factors located in the proximal region (bp –57 to –50) of the promoter. This sequence binds the myeloid-specific factor PU.1 and the hematopoietic-associated factor complex HAF-1 (16–19). HAF-1 contains the interferon response factors IRF-1 and ICISBP and the ets family members Elf-1 or PU.1. The importance of this ets binding site is evidenced by the detection of single base pair mutations in this sequence in some kindreds with chronic granulomatous disease (17–19), a genetic disorder characterized by a non-functional phagocyte NADPH oxidase, impaired microbial function, and recurrent severe infections.

Our recent studies indicate that ets factor cis elements also play critical roles in the transcriptional regulation of both the p47phox and p40phox genes. In p47phox, a single consensus PU.1 binding site located on the noncoding strand from bp –40 to –45 (relative to the transcription start site) accounted for most of the myeloid-specific promoter activity found in the proximal 2515 base pairs of the 5′-flanking region of the gene (20). Mutation of this site essentially abolished p47phox promoter/reporter gene activity in myeloid cells. Furthermore, we

* This work was supported by National Institutes of Health Grant AI20866. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ To whom correspondence should be addressed: Dept. of Medicine, University of Texas Health Science Center, 7703 Floyd Curl Dr., San Antonio, TX 78229-3900. Tel.: 210-567-4810; Fax: 210-567-4654; E-mail: clarkra@uthscsa.edu.

1 The abbreviations used are: bp, base pair(s); C/EBP, CCAAT/enhancer binding protein; ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; PCR, polymerase chain reaction; PIPES, 1,4-piperazinediethanesulfonic acid; CBP, CREB binding protein; CMV, cytomegalovirus.
showed that this site bound PU.1 with high avidity, and mutations of flanking sequences that reduced the avidity of PU.1 binding also resulted in a proportionate reduction in promoter activity (21). In p40phox, we analyzed ~6000 bp of the gene and identified three PU.1 binding sites that directed gene transcription in a myeloid-specific manner.2 Two sites are located in the proximal 120 bp of the 5′-flanking sequence of the gene, and the third is in the putative 5′ untranslated region of the transcript. Mutation analysis indicated that each site contributed to a different extent to the overall promoter activity, and mutation of all three sites abolished promoter activity in myeloid cells. In accord with our mutation studies on the p47phox promoterless luciferase reporter plasmid pGL3-Basic at the same restriction sites. Site-directed mutagenesis was carried out using the QuikChange kit (Stratagene, La Jolla, CA). The mutated nucleotides are shown in italics in Fig. 1. All constructs were confirmed by restriction mapping and sequencing.

Cell Culture—The human promyelocytic cell line HL-60 and the myeloid leukemia cell line PLB-985 were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 10 μM HEPES. The human cervical carcinoma epithelial cell line HeLa, the lymphoid leukemia cell line Jurkat, and the human colon epithelial cell lines LoVo and HCT-116 were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. All media contained penicillin and streptomycin.

Transient Transfections—For suspension cultures, transfection was carried out by electroporation when cells reached a density of about 5 × 10^6 cells/ml. Cells were resuspended in medium containing 20 μg of the luciferase reporter constructs and 0.5 μg of a Renilla luciferase vector (pRL-null, Promega) as a transfection efficiency control. Electroporation was carried out at 960 μF and 250 V. Unless otherwise indicated, at 8 h, the cells were washed twice 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 dual luciferase assay system (Promega) using a Turner Designs TD-2020 luminometer. For adherent cells, transfections were done using LipofectAMINE Plus reagent (Life Technologies, Inc.) according to the manufacturer's protocol.

Nuclear Extracts—HL-60 cells were disrupted by cavitation using a technique described previously for polymorphonuclear leukocytes (23). The cells were washed twice in phosphate-buffered saline, 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 mM diisopropyl fluorophosphate (Sigma) added. The cells were kept on ice for 10 min and then centrifuged at 400 × g for 5 min. The cell pellet was resuspended in 10 ml of relaxation buffer, 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 EGTA, 100 mM MgCl2, 20 mM dithiothreitol, 4 mM phenylmethylsulfonyl fluoride, and 2 mM sodium orthovanadate. The cavitaded cells were centrifuged at 400 × g for 10 min at 4 °C, and the nuclear-enriched pellet was resuspended and further purified on a discontinuous gradient of sucrose (0.3/0.88M). The nuclear fraction was pelleted at 400 × g, and the supernatant was saved for EMSA.

DNase I Protection Assay—A fragment of DNA corresponding to the p67phox promoter region between −731 and −635 was labeled on the noncoding strand by successive T4 polynucleotide kinase and polymerase chain reaction. Briefly, 10 fmol of the reverse-complemented oligonucleotide (5′-GGCTTTTACCAATTGAAAT-3′) was incubated at 37 °C for 30 min in a 10-μl volume containing 30 μl of (−23P)ATP, 1× PCR buffer, and 5 units of Taq DNA polymerase (Promega). The kinase was inactivated by heating at 65 °C for 20 min, and the reaction was adjusted to 1× PCR buffer; 2 μg of MGO1; 125 μM each dATP, dCTP, and dTTP, and dUTP; 10 fmol of the forward primer 5′-gagctcGTTTTTCTATACCTCT-3′ with 50 ng of the template DNA (pGL3-p67-330); and 1 unit of T4 polymerase (Promega) in a final volume of 50 μl. Thirty cycles of PCR were carried out (94 °C for 30 s, 54 °C for 30 s, and 72 °C for 30 s), and the ~100-bp product was purified by agarose gel electrophoresis. The activity of the DNA preparation was ~10^6 cpm/μl. Nuclear extracts were prepared from HL-60 cells as noted above.

Electrophoretic Mobility Shift Assay (EMSA)—Complementary DNA oligonucleotides were annealed by heating in 1× NET at 95 °C for 5 min and cooling at ambient temperature. Probes were then labeled with (−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/μl bovine serum albumin, and 2 μg of poly(dI-dC). For supershift assays, 2 μg of specific antibody were added, and the reaction was continued for 30 min. Samples were loaded 5% nondenaturing polyacrylamide gels, and electrophoresis was carried out at 4 °C 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 way, except that the reaction mixture was preincubated with competitor DNA for 10 min at 4 °C before addition of the labeled probe.

Chromatin Immunoprecipitation (ChIP) Assay—Formaldehyde

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2 S. L. Li, A. J. Valente, W. Schlegel, M. J. Gamez, and R. A. Clark, submitted for publication.
cross-linking and immunoprecipitation of chromatin were carried out as described by Farnham and co-workers (24). Briefly, formaldehyde was added directly to cell cultures to a final concentration of 1%, the cells were incubated at 25 °C for 10 min, and then glycine was added to stop the fixation. Cells were collected by centrifugation and washed with phosphate-buffered saline containing 0.5 mM phenylmethylsulfonyl fluoride. Nuclei were collected, resuspended in 50 mM Tris-HCl (pH 8.1), 1% SDS, 10 mM EDTA, plus phenylmethylsulfonyl fluoride, leupeptin, and antipain and incubated on ice for 10 min. Nuclei were then sonicated on ice for 10 min. Samples were sonicated on ice to break chromatin DNA to an average length of ~600 bp and then treated with proteinase K. Samples were then precipitated, resupended, and treated with proteinase K. Samples were then pelleted from formalin-fixed Staph A cells (Roche Molecular Biochemicals). Precleared chromatin from 2 × 107 cells was incubated alone or with 1.5 µg of affinity-purified rabbit polyclonal antibody (Santa Cruz Biotechnology) and rotated at 4 °C for 12–16 h. 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, beginning at the cross-link reversal step. Cross-links were reversed by incubation at 65 °C for 5 h in the presence of 300 mM NaCl. During this process, RNA was digested by 0.01% RNase A. Samples were then precipitated, resuspended, and treated with proteinase K. The activity of these constructs was assayed in the HL-60 human myeloid cell line by transient transfection. As shown in Fig. 2, a ~30-fold increase in luciferase reporter gene activity was observed in lysates prepared from cells transfected with pGL3-p67–733 and pGL3-p67–862, compared with pGL3-Basic vector. Constructs extending further upstream gave progressively less luciferase activity, suggesting the presence of negative regulatory elements between bp –862 and –2022. However, a deletion to nucleotide –682 resulted in >80% reduction in activity, indicating that sequences important for p67phox promoter activity are located between bp –733 and –682.

To identify the potential protein-binding sites in this active region, a DNase I protection assay was carried out using nuclear extracts from HL-60 cells and a 32P-labeled fragment of DNA corresponding to the region of –733 to –637 of the p67phox gene. A broad protected region corresponding to the 25 nucleotide sequence CTCAGAATGGGGGGCGAGGAC located at –704 to –680 was identified (Fig. 3). The core of this sequence, GGAGGG (~94 to ~688), is very similar to the consensus binding sequence (GGGGCGG) for the Sp1 family of ubiquitously expressed transcription factors.

To determine whether the p67phox sequence could bind Sp1 factors, EMSA was carried out using a labeled oligonucleotide corresponding to the protected region of the gene. This oligonucleotide formed a number of DNA-protein complexes with nuclear extract from HL-60 cells (Fig. 4A). A pattern characteristic of Sp1 factors binding to their cognate element was observed, and this was confirmed by competition with both

**RESULTS**

A functional Sp1 Site Is Present in the Proximal 5'-Flanking Sequence of the Human p67phox Gene—The promoter region of p67phox was cloned using the PromoterFinder kit (CLONTECH). The specific PCR product amplified from the genomic library appeared as a single band (~1.5 kilobases) on agarose gel. This amplicon was cloned directly into the pCRIII vector (Invitrogen), and its identity was confirmed by the overlapping sequence between the 3' end of the clone and the known 5' end of p67phox cDNA (25, 26). A clone was sequenced and later found to be identical to the sequence of the p67phox gene deposited in the GenBank™ (accession number NM_005003 (51550–533640)), except for a G to A change at bp –912 and the deletion of 3 of 18 tandem A residues between bp –1171 and –1169 (nucleotide positions relative to the ATG translation initiation codon). The sequenced clone was subcloned into the luciferase reporter vector pGL3-Basic. Based on this plasmid, a series of deletion constructs was made, all of which extended downstream to nucleotide –534 in exon 1 of the p67phox gene (Fig. 1). The activity of these constructs was assayed in the HL-60 human myeloid cell line by transient transfection. As shown in Fig. 2, a ~30-fold increase in luciferase reporter gene activity was observed in lysates prepared from cells transfected with pGL3-p67–733 and pGL3-p67–862, compared with pGL3-Basic vector. Constructs extending further upstream gave progressively less luciferase activity, suggesting the presence of negative regulatory elements between bp –862 and –2022. However, a deletion to nucleotide –682 resulted in >80% reduction in activity, indicating that sequences important for p67phox promoter activity are located between bp –733 and –682.

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amplification by genomic primers specific for this region of the p67phox DNA was purified and used as the template for PCR. Anti-Sp3 antiserum. Cross-linking was reversed, and the re-in vivo assay was performed using an antibody to the transcription factor Sp3 protein in HL-60 cells binds to the p67phox promoter. As a control for antibody specificity, we carried out the assay using an antibody to the transcription factor Sp1 family of proteins bind in vivo to this cis element, we performed a ChIP analysis on the HL-60 cells. DNA-protein complexes were cross-linked in vivo by the addition of formaldehyde to the cultures, and the chromatin DNA was sheared and immunoprecipitated with anti-Sp3 antiserum. Cross-linking was reversed, and the released DNA was purified and used as the template for PCR amplification by genomic primers specific for this region of the p67phox promoter. As a control for antibody specificity, we carried out the assay using an antibody to the transcription factor C/EBPβ, which is expressed in HL-60 cells but does not have a cognate binding site in this region of the p67phox promoter. As a control for PCR specificity, we used p40phox primers designed to amplify a region of the p40phox gene that does not contain Sp1 or C/EBPβ binding sites. The PCR primers specific for p67phox produced an amplicon of predicted size (317 bp) from both the total input DNA and the anti-Sp3 immunoprecipitates but not from the anti-C/EBPβ immunoprecipitates (Fig. 4B). Furthermore, the p40phox primers produced an amplicon only from the total input DNA. These data indicate that endogenous Sp3 protein in HL-60 cells binds to the p67phox promoter and that Sp3 or Sp1 factors may promote transcription of the p67phox gene in vivo.

The First Intron Is Essential for Myeloid-specific Activity of the p67phox Promoter—Although the reporter gene constructs described above showed promoter activity, it was not restricted to cells of myeloid lineage. These constructs were active in several cell types, including the nonmyeloid cell lines LoVo and HCT-116. To locate the DNA sequences that direct myeloid-specific expression of the p67phox gene, we extended the promoter sequences in both directions. PCR primers were designed based on the sequence of the p67phox gene deposited in GenBank™ (NM005003), and the genomic DNA libraries in the PromoterFinder kit were used as templates. Initially, we kept the 3' end of the p67phox promoter fragment unchanged at bp −534 and extended the 5' end to bp −3669, producing the construct pGL3-p67−3669. It was reasonable that this construct might dictate myeloid-specific expression of the reporter luciferase gene, because it contained two strong binding sites for the myeloid-specific transcription factor PU.1, one at bp −3291 and the other at −2584, which were identified by sequence analysis and EMSA (data not shown; also refer to Fig. 6D). However, the pGL3-p67−3669 construct was 30–50% less active than pGL3-p67−2022 and was functional in both myeloid (HL-60 and PLB-985) and nonmyeloid (LoVo and HCT-116) cell lines (data not shown).

We then analyzed the downstream sequences for cis elements that might mediate myeloid-restricted expression. A segment of the p67phox gene extending from bp −4 to bp −3624 (relative to ATG) was obtained by PCR amplification. This DNA segment, composed of the 5' flanking sequence, exon 1, intron 1, and 27 bp of exon 2 of the gene, was subcloned into the pGL3-Basic reporter vector as before. This reporter construct was designated pGL3-p67−3624. Two 5' deletion constructs that extended to bp −985 (291 bp 5' to the active Sp1 site) and bp −678 (16 bp 3' to the active Sp1 site) were also made for comparison. The highest luciferase activity was observed in HL-60 lysates prepared from cells transfected with construct pGL3-p67−985, compared with pGL3-Basic vector (Fig. 5). Further extension from bp −985 to −3624 (pGL3-p67−3624) led to a 65% decrease in the luciferase activity. Deletion construct pGL3-p67−678, which excluded the Sp1 site, showed a 55%
for IFN-γ induction of p67phox gene expression in the myeloid cell line U937. A sequence between –196 and –164 (AAAGGT- GGAGACATTCTCTGATGATTTGCCAC) was found to be responsible for this induction and was identified as a HAF1 site, because it binds a trimolecular HAF1 complex (composed of PU.1 or Elf1, ICSBP, and IRF-1). We will refer it as the PU.1/HAF1 site because this sequence also binds PU.1 protein independently (see below). Inspection of the intron 1 sequence revealed two additional well defined cis-acting elements, an AP-1 site (TGAGTCA) located at –210 and a second PU.1 site located at –289 (Fig. 1). We further investigated the role of these binding sites in p67phox gene transcription.

Transcriptional Activation of the p67phox Promoter by PU.1 Is Influenced by the Location of the PU.1 Binding Site—To determine whether the PU.1 sites within intron 1 of the p67phox gene bind PU.1 protein, we performed EMSA using HL-60 nuclear extract. A DNA-protein complex was formed between 32P-labeled double-stranded oligonucleotide probe encompassing the PU.1(1)/HAF1 site and the nuclear extract (Fig. 6A). This complex was supershifted by PU.1 antibody but not by IgG, demonstrating that endogenous PU.1 protein binds to this site of the p67phox promoter. However, the downstream PU.1 site, designated PU.1(1)/HAF1 in Fig. 1, exhibited more complexity. Oligonucleotide probes derived from this site formed several bands of DNA-protein complexes on EMSA (Fig. 6B), most of which could be considered specific, because their formation was inhibited by excess unlabeled probe. The two lower bands contained PU.1 or its degradation products because they were abrogated by PU.1 antibody. On the other hand, the two upper bands were supershifted by Elf1 antibody, indicating that this alternative member of the elf family was a component of the complexes. The latter observation is reminiscent of reports that Elf1, rather than PU.1, is present in complexes binding the HAF1 site in the gp91phox promoter (18). Finally, the relative binding affinity of these PU.1 sites was compared with the previously characterized p47phox promoter PU.1 site, using cold competition EMSA. As shown in Fig. 6C, p67-PU.1(2) and
FIG. 6. All four potential PU.1 sites of the p67phox promoter bind PU.1 protein in vitro and in vivo. A, EMSA of HL-60 nuclear extracts with 32P-p67-PU.1(2) oligonucleotide probe. The probe was incubated with the nuclear extract (6 μg) alone (lane 1) or together with either antibody to PU.1 (lane 2), control IgG (lane 3), or a 200-fold molar excess of the homologous unlabeled oligonucleotide (lane 4). The specific PU.1-DNA complex (PU.1) and the supershifted complex (SS) are indicated by arrows. B, EMSA of HL-60 nuclear extracts with 32P-p67-PU.1(1)/HAF1 oligonucleotide probe. The probe was incubated with the nuclear extract alone (lane 1) or together with either antibody to PU.1 (lane 3), Elf1 (lane 4), or a 200-fold molar excess of the homologous unlabeled oligonucleotide (lane 2). HAF1 and HAF1a indicate faster- and slower-migrating HAF1-like complexes, respectively. C, the PU.1 binding affinities of the p67phox promoter PU.1(1)/HAF1 and PU.1(2) sites compared with the p47phox promoter PU.1 site. 32P-p47-PU.1 was incubated with HL-60 nuclear extract alone (left lane) or together with a 10-, 100-, or 1000-fold molar excess of unlabeled oligonucleotide corresponding to p47-PU.1, p67-PU.1(1), or p67-PU.1(2) sites, as indicated. D, comparison of PU.1 binding affinities among the four PU.1 sites of the p67phox promoter. 32P-p67-PU.1(4) probe was incubated with HL-60 nuclear extract alone (lane 1) or together with a 100- or 1000-fold molar excess of unlabeled oligonucleotide probes of either p67-PU.1(1), p67-PU.1(2), p67-PU.1(3), or p67-PU.1(4) sites, as indicated. E, ChIP analysis of the p67phox promoter PU.1 binding sites. Cross-linked HL-60 chromatin was immunoprecipitated with antibodies to either PU.1 or C/EBPα, or in the absence of antibody (input). Cross-linking was reversed, and the DNA was purified and analyzed by PCR with primers specific for each of the PU.1 binding sites of the p67phox promoter or for the CCR5 promoter.
PU.1(1)/HAF1 bound to PU.1 protein in HL-60 nuclear extracts only weakly, being 100- and 1000-fold less active, respectively, than the p47–PU.1 site. The binding affinities of p67–PU.1(2) and PU.1/HAF1 were also compared with the apparently nonfunctional PU.1(3) and PU.1(4) sites described above. Competition EMSA showed that the upstream PU.1 sites possessed much stronger PU.1 binding affinity than those within the first intron (Fig. 6D). This result was not unexpected based on our previous study (21) showing that the (G/C)T residues flanking the 3′ end and the tandem T or A at the 5′ end of the PU.1 binding consensus sequence GAGGAA are required for optimal PU.1 binding ability.

Using ChIP analysis, we then investigated whether PU.1 protein binds to these PU.1 sites in vivo. The assay was carried out in HL-60 cells as before, except that antibody specific to PU.1 was used to immunoprecipitate the cross-linked chromatin DNA. As shown in Fig. 6E, PCR amplicons from the regions containing PU.1(4), PU.1(3), and both PU.1(2) and PU.1(1)/HAF1 were obtained with the PU.1 immunoprecipitated chromatin, but not with the negative control C/EBPα immunoprecipitates. The PCR negative control, a DNA fragment from the CCR5 promoter that does not contain either PU.1 or C/EBPα binding sites, was amplified only from the input DNA. These data demonstrate that endogenous PU.1 protein binds to the p67phox promoter at the indicated sites in vivo.

We next tested the role of these PU.1 sites in p67phox promoter activity by transient transfection assays. Mutation of either the PU.1(2) or PU.1(1)/HAF1 site in the pGL3–p67–985 construct reduced the p67phox promoter activity by ∼50% in HL-60 cells (Fig. 7A), but produced no significant difference in promoter activity in HeLa or Jurkat cells, indicating the importance of these PU.1 sites and their binding to PU.1 protein for expression of the p67phox gene in myeloid cells. On the other hand, mutations of the PU.1(3) and PU.1(4) sites in the pGL3–p67–3624 construct produced no reduction of promoter activity (Fig. 7B). We conclude that p67phox transcriptional activation by PU.1 is dependent on the location of the PU.1 binding site.

AP-1 Is Critical for p67phox Promoter Activity—A TFSEARCH search analysis (27) identified an exact consensus AP-1 site, ATGAGTCAG, located between bp −211 and −203. When a labeled oligonucleotide probe derived from this site was used in EMSA, a specific band was observed with HL-60 nuclear extract. This complex could be inhibited with excess wild-type but not mutated (TGGATCA to TGAGTTG) probes (Fig. 8A). The identity of the AP-1 proteins forming this complex was investigated by supershift assays using specific antibodies. A broadly reactive antibody to the Jun family of AP-1 proteins (c-Jun/AP-1) effectively abolished the formation of the DNA-protein complex, indicating that the Jun proteins were probably a component of all the dimeric complexes binding this sequence (Fig. 8A). Antibodies specific for the individual members of the Jun family showed that c-Jun, Jun B and Jun D formed complexes with the p67phox AP-1 site, Jun D being the most abundant and c-Jun the least abundant. Antibody to the Fos family of proteins greatly reduced but did not abolish the specific complex, suggesting that the binding proteins in the HL-60 nuclear extract were made up predominantly of Jun/Fos heterodimers and a small amount of Jun/Jun family dimers. Antibody to CREB-1 and ATF-2, like the irrelevant anti-GATA-6 and the negative control IgG, had no effect on the EMSA reaction.

To investigate whether AP-1 factors bound this site in vivo, a ChIP assay was carried out with the antibodies broadly reactive with c-Fos or c-Jun family proteins. As a negative control, we included an immunoprecipitation reaction with the C/EBPα antibody. As shown in Fig. 8, B and C, the p67-intron 1 primers produced a 353-bp amplicon, as predicted, from the input as well as from the anti-c-Fos and anti-c-Jun immunoprecipitated DNA, but not from the anti-C/EBPα immunoprecipitates (Fig. 8, B and C). The PCR control p40–PU.1 primers produced a PCR product only from the input DNA. The p40–PU.1 primers were designed to amplify a region of the p40phox promoter that does not contain consensus AP-1 or C/EBPα binding sites. These data indicate that Fos and Jun proteins in HL-60 cells bind to the p67phox promoter, most abundantly as heterodimers, and thus may promote p67phox gene transcription in vivo.

The influence of the AP-1 site on p67phox promoter activity was investigated in the HL-60 cells. Mutation of the AP-1 site alone in the strongly active pGL3–p67–985 construct (∼700-fold increase over empty vector) effectively abolished promoter activity (Fig. 9A). This result was surprising, because we had observed that the Sp1 and the PU.1(1)/HAF1 and PU.1(2) sites also contributed to the overall activity to the construct. To determine the contribution of the AP-1 site alone, the construct pGL3–p67−405−3 M, bearing mutations at the PU.1(1)/HAF1, PU.1(2), and Sp1 sites, was investigated. The decrease in activity was greater than that caused by the sum of mutations of each site alone, but this reduction was not as complete as that seen with the AP-1 mutation alone. When all four cis elements

**Fig. 7.** Mutations of PU.1(1) and PU.1(2) in the first intron of p67phox, but not of PU.1(3) and PU.1(4) in the distal p67phox promoter, specifically reduce promoter activity in myeloid cells. A, the pGL3–p67–985–4 construct was mutated at the PU.1(1) and PU.1(2) sites (see Fig. 1) and transfected into HL-60, HeLa, or Jurkat cells. Luciferase activity was assayed as described in Fig. 3. B, the pGL3–p67−3642−4 construct was mutated at the PU.1(3) or PU.1(4) sites (see Fig. 1) and transfected into HL-60 cells. Luciferase activity was assayed and the results were expressed as in Fig. 5. The data shown (mean ± S.E.) are from at least three independent experiments.
To confirm that AP-1 can transactivate p67phox promoter activity, co-transactivation experiments were performed in F9 embryonal carcinoma cells, which lack endogenous AP-1 activity and express only very low levels of Fos/Jun proteins. The promoterless luciferase reporter vector pGL3-Basic and the p67phox luciferase constructs pGL3-p67-985, pGL3-p47-985-AP1mut and pGL3-p67-985-4m (all four identified sites mutated) were co-transfected into F9 cells with either the Jun B expression plasmid (CMV Jun B) or with the empty vector (Fig. 9B). Co-expression of Jun B increased the activity of the p67phox promoters about 3-fold, whereas there was no such increase in the same construct with a mutated AP-1 site or four mutated sites. These results show that Jun proteins can transactivate the p67phox promoter through the AP-1 site identified in the intron 1 region. The modest increase in transacting activity that was observed might reflect our earlier observations that the pGL3-p47-985 is only very weakly active in nonmyeloid cells. It could also be explained by the fact that only a very small pool of Fos proteins is available in the F9 cells. This would limit the formation of Fos/Jun heterodimers, which may be more active than Jun B/Jun B homodimers in activating the p67phox promoter.

**DISCUSSION**

In previous studies, we demonstrated an essential role for a single ets family transcription factor, PU.1, in the transcriptional regulation of both the p40phox and p47phox genes (20). In contrast, the studies presented here show that transcription of the p67phox gene is regulated by a diverse array of factors, including AP-1, Sp1, PU.1, and the complex HAF-1. Moreover, our transfection studies indicate that it is those factors that bind the AP-1 site, rather than PU.1, that are essential for the functional activity of the promoter. Supershift analysis using specific antibodies and HL-60 nuclear extract identified members of the Jun and Fos family of transcription factors that bind this critical AP-1 site.

The region of the gene encoding the 5'-UTR of the p67phox transcripts is interrupted by a 480-bp intronic sequence (intron 1) starting at bp -510 relative to the translation initiation codon. Previous studies had provided evidence that p67phox transcription may be initiated from a number of sites, including at least one in the first intron (19, 25, 26). Our initial studies of the p67phox promoter were focused on the 5'-flanking region of the gene and the first series of promoter deletion constructs all terminated downstream at bp -534, thus including a number of these putative transcription initiation sites, but excluding intron 1. The longest construct in this series extended from position -3669 and included two potential PU.1 sites (PU.1(3) and PU.1(4), Fig. 1), which were identified based on our previously defined criteria for optimal flanking sequences (21). In gel shift assays, both of these sites were shown to bind PU.1 in vitro, and ChIP analysis indicated that they were occupied by PU.1 in vivo. However, this construct demonstrated strong promoter activity in both myeloid and nonmyeloid cell lines, indicating that these distal PU.1 sites did not direct myeloid-specific promoter activity. Furthermore, mutation of these sites, which abolished PU.1 binding, did not affect the activity of the reporter gene constructs, suggesting that the distal PU.1 sites contributed minimally to overall promoter activity. Deletion studies mapped the most potent promoter activity in these constructs to a region between -733 and -682 bp, in which a strong binding site (−694 to −688) for the ubiquitous Sp1 family of factors was identified by both DNase I footprint and gel shift analysis. Deletion or mutation of this Sp1 site resulted in a nearly complete loss of activity in these initial reporter gene constructs.

While these studies were in progress, Eklund and Kakar (19)
reported that IFN-γ-induced expression of p67phox in myelomonocytic U937 cells was mediated by a PU.1 and Sp1 sites reducing p67phox promoter by >90%. The indicated mutations (also see Fig. 1) were introduced into the pGL-p67–678/-4 or pGL-p67–985/-4 constructs by site-directed mutagenesis. Transfection of HL-60 cells, determination of luciferase activity, and expression of results were as described in Fig. 5. B, coexpression of Jun B transactivates the p67phox promoter in F9 cells. Two hundred ng of Jun B expression plasmid (CMV Jun B) or the empty vector (CMV vector) were co-transfected into F9 cells with 200 ng of the indicated wild-type, mutant AP-1 (AP-1Mt), or multiple site-mutated (AP-1/Sp1/PU.1(1)-(2)Mt) reporter constructs. Determination of luciferase activity and expression of results were as described in Fig. 5.

**Fig. 9.** Among the four active promoter elements, the AP-1 site is essential for the p67phox promoter activity in myeloid cells. A, mutation of the AP-1 site alone or in combination with mutations of the PU.1 and Sp1 sites reduces p67phox promoter by >90%. The indicated mutations (also see Fig. 1) were introduced into the pGL-p67–678/-4 or pGL-p67–985/-4 constructs by site-directed mutagenesis. Transfection of HL-60 cells, determination of luciferase activity, and expression of results were as described in Fig. 5. B, coexpression of Jun B transactivates the p67phox promoter in F9 cells. Two hundred ng of Jun B expression plasmid (CMV Jun B) or the empty vector (CMV vector) were co-transfected into F9 cells with 200 ng of the indicated wild-type, mutant AP-1 (AP-1Mt), or multiple site-mutated (AP-1/Sp1/PU.1(1)-(2)Mt) reporter constructs. Determination of luciferase activity and expression of results were as described in Fig. 5.

**AP-1 Regulates p67phox Promoter**

The identified AP-1, Sp1, PU.1, and PU.1/HAF-1 sites in the pGL3-p67–985/-4 construct effectively abolished its activity in HL-60 cells, indicating that these sites are the major elements regulating transcription in this myeloid cell line. Mutation of either the Sp1, PU.1, or PU.1/HAF-1 sites alone each reduced promoter activity by 30 to 50%. However, mutation of the AP-1 site alone resulted in greater than 90% reduction in activity. Thus, the AP-1 site appears to be essential for the function of the promoter. However, because the activity mediated by the AP-1 site alone was weak when transfected into HL-60 cells, factors binding this site appear to be acting cooperatively or synergistically with those binding to other sites.

Regulation of transcription through cooperation between AP-1 proteins and members of the ets family of protooncogenes has been reported for several genes. It was originally shown that the ets factors c-ets-1 and c-ets-2 could cooperate with AP-1 factors to activate transcription through a combined AP-1/PEA3 site in the polyoma enhancer. (28) Similar cooperativity was described in the transcription of genes such as uPA (AP-1 and ets-2), interleukin 3 (AP-1 and Elf-1), and granulocyte-macrophage colony-stimulating factor (AP-1 and Elf-1), among others (29–32). In these examples, cooperative transactivation is mediated by a combined AP-1/ets binding motif in which the AP-1 site is located im-

**FIG. 9.** Among the four active promoter elements, the AP-1 site is essential for the p67phox promoter activity in myeloid cells. A, mutation of the AP-1 site alone or in combination with mutations of the PU.1 and Sp1 sites reduces p67phox promoter by >90%. The indicated mutations (also see Fig. 1) were introduced into the pGL-p67–678/-4 or pGL-p67–985/-4 constructs by site-directed mutagenesis. Transfection of HL-60 cells, determination of luciferase activity, and expression of results were as described in Fig. 5. B, coexpression of Jun B transactivates the p67phox promoter in F9 cells. Two hundred ng of Jun B expression plasmid (CMV Jun B) or the empty vector (CMV vector) were co-transfected into F9 cells with 200 ng of the indicated wild-type, mutant AP-1 (AP-1Mt), or multiple site-mutated (AP-1/Sp1/PU.1(1)-(2)Mt) reporter constructs. Determination of luciferase activity and expression of results were as described in Fig. 5.
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