Critical Flanking Sequences of PU.1 Binding Sites in
Myeloid-specific Promoters*

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The myeloid-specific transcription factor PU.1 is essential for expression of p47\textsuperscript{phox}, a component of the superoxide-forming phagocyte NADPH oxidase. The consensus PU.1 binding sequence (GAGGAA) is located on the non-coding strand from position −40 to −45 relative to the transcriptional start site of the p47\textsuperscript{phox} promoter. A promoter construct extending to −46 was sufficient to drive tissue-specific expression of the luciferase reporter gene, but extension of the promoter from −46 to −48 resulted in a significant increase in reporter expression. Mutations of the nucleotides G at −46 and/or T at −47 reduced both reporter expression and PU.1 binding, whereas mutations at −48 had no effect. The PU.1 binding avidity of these sequences correlated closely with their capacity to dictate reporter gene transcription. In parallel studies on the functional PU.1 site in the promoter of CD18, mutations of nucleotides G and T at positions −76 and −77 (corresponding to −46 and −47, respectively, of the p47\textsuperscript{phox} promoter) reduced PU.1 binding and nearly abolished the contribution of this element to promoter activity. We conclude that the immediate flanking nucleotides of the PU.1 consensus motif have significant effects on PU.1 binding avidity and activity and that this region is the dominant cis element regulating p47\textsuperscript{phox} expression.

During myelopoiesis pluripotent hematopoietic stem cells become committed as myeloid precursor cells and differentiate into morphologically and functionally distinct end-stage neutrophils, eosinophils, and monocyes. Myeloid development is regulated by a combination of hematopoietic growth factors, growth factor receptors, and lineage-restricted transcription factors (1). The myeloid-specific transcription factor PU.1, the most divergent member of the \textit{ets} family of transcription factors (2, 3), is required for terminal myeloid differentiation and gene expression (4). PU.1 contacts DNA with a novel loop-helix-loop architecture, and binds to a \textit{cis} element with a core sequence 5’-GAGGAA-3’ (5, 6). This element is sometimes clustered with binding sites for other transcription factors, which may facilitate interactions among these different trans-acting factors. Examples include the PIP\textsuperscript{1} site within the immunoglobulin light chain gene enhancers Ex8, EY2, and EY3, and the FEF site in the c-fes promoter and chicken lysozyme enhancer (7, 8). Whereas PU.1 binding is dispensable for FEF-DNA interaction, it is a prerequisite for PIP participation in the PU.1-PIP-DNA complex (7, 8).

PU.1 binds a number of myeloid cell-restricted target genes, such as p47\textsuperscript{phox} (9), gp91\textsuperscript{phox} (10), macrophage colony-stimulating factor receptor (M-CSFR) (11), granulocyte colony-stimulating factor receptor (G-CSFR) (12), \textit{F}c-receptor (13), scavenger receptor (14), and integrin subunits CD11b (15) and CD18 (16). CD18 is the \(\beta\)2 subunit of the leukocyte integrins, a family of cell surface proteins that mediate leukocyte adhesion and thereby play a critical role in the inflammatory response (17, 18). The importance of the leukocyte integrins in inflammation is illustrated by leukocyte adhesion deficiency syndrome, a rare inherited disorder caused by mutations in the CD18 gene and associated with severe and recurrent infections (17, 18). The products of the p47\textsuperscript{phox} and gp91\textsuperscript{phox} genes are components of the phagocyte NADPH oxidase, which by generating superoxide anion serves as a pivotal enzyme in the microbicidal function of phagocytic cells (19, 20). The importance of the phagocyte oxidase is demonstrated by chronic granulomatous disease (CGD), an inherited disorder in which a functionally defective NADPH oxidase results in severe and recurrent infections (19–22).

We previously isolated the p47\textsuperscript{phox} promoter and showed that it contains a consensus PU.1 binding sequence on the non-coding strand from base pair −40 to −45 relative to the transcriptional start site (9). Mutation of this sequence abolishes PU.1 binding and promoter activity. Although a p47\textsuperscript{phox} promoter-luciferase reporter construct extending to −46 could dictate tissue-specific expression in myeloid cells, a larger construct (−86) showed maximal promoter activity, implying a significant contribution of upstream sequences to the expression of p47\textsuperscript{phox}. Since in DNase I footprint analysis, a protected region (−37 to −52) was observed to extend beyond the consensus sequence GAGGAA (−40 to −45), the current studies were performed to define the functional role of the flanking residues upstream of the PU.1 site.

EXPERIMENTAL PROCEDURES

Materials—RPMI 1640 was obtained from Life Technologies, Inc. Restriction enzymes, T4 polynucleotide kinase, pGL3-Basic luciferase vector, and dual-luciferase reporter assay kit were from Promega (Madison, WI). [γ\textsuperscript{32}P]ATP (6000 Ci/mm) was obtained from NEN Life Science Products. Oligonucleotides were synthesized and DNA sequenced by the Advanced DNA Technology Unit, University of Texas Health Science Center, San Antonio, TX. Polyclonal PU.1 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

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‡ The abbreviations used are: PIP, PU.1 interaction partner; CGD, chronic granulomatous disease; EMSA, electrophoretic mobility shift assay; PEF, c-fes expression factor; PCR, polymerase chain reaction; TSS, transcriptional start site; PIPES, 1,4-piperazinediethanesulfonic acid.
Luciferase Vectors—Reporter vectors in the pGL3-Basic luciferase plasmid including pGL3-p47–86, pGL3-p47–48, and pGL3-p47–36 (i.e., the proximal p47phox promoter extending to positions −86, −48, −46, and −36, respectively, relative to the TSS) were constructed as described previously (9). Briefly, PCR was carried out using the pGL3-p47–127 as template, a luciferase antisense oligonucleotide (pGL primer 2; 5′-CTTATAGTTTGGCGTTCCTC-3′) as the reverse primer and oligonucleotides synthesized with an XhoI restriction site linked to the desired 5′ terminus of the p47phox promoter as the forward primers. For CDIS analysis, CD18(0.9)/luc (the generous gift of Dr. A. Rosmarin, Brown University, Providence, RI) and the pCR primer were used to amplify a pGL3-Basic plasmid construct pGL3-p47–127 as template, a luciferase antisense oligonucleotide (pGL primer 2; 5′-CTTATAGTTTGGCGTTCCTC-3′) as the reverse primer and oligonucleotides synthesized with an XhoI restriction site for the p47phox promoter and Renilla activity using the dual-luciferase assay system (Promega) and a Turner TD-20/20 luminometer.

In Vitro Translation—The mouse PU.1 cDNA (generous gift of Dr. M. Klemsz, Indiana University, Indianapolis, IN) (2) was excised by digestion with EcoRI and ligated into the pBluescript plasmid. 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 PCR-amplified products were digested with XhoI and HindIII and cloned into the corresponding sites of pGL3-Basic. To generate mutated constructs, altered oligonucleotides were used as the forward primers. The inserts of the p47phox constructs all extended downstream to +52 relative to the TSS and used the p47phox translation initiation codon ligated in-frame to the luciferase open reading frame. The CDIS promoter constructs extended downstream to +27 and used the translation initiation codon of the luciferase open reading frame.

Transient Transfections—THP-1 cells were maintained at a density of ~10^6 cells/ml and for transfection were then resuspended in medium containing 20 μg of the luciferase reporter constructs and 2 μg of the pRL-CMV plasmid (Promega) as a transfection efficiency control. Electroporation was carried out at 960 V and 250 V. After 48 h the cells were washed three times in phosphate-buffered saline, pH 7.4, lysed in 100 μl of reporter lysis buffer, microcentrifuged for 5 min, and 20-μl aliquots of the supernatant assayed for both luciferase and Renilla activity using the dual-luciferase assay system (Promega) and a Turner TD-20/20 luminometer.

Nuclear Extracts—THP-1 cells were disrupted by cavitation using a technique described previously for neutrophils (24). Briefly, 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 mM diisopropylphosphoryl fluoride (Sigma) were added. The cells were kept on ice for 10 min, then centrifuged at 400 g for 10 min, then centrifuged at 4 °C and the nucleus-enriched pellet resuspended and further purified on a discontinuous gradient of sucrose (0.3/0.88 M). The nuclear fraction was extracted in 100 μl of urea extraction buffer (1 M urea, 1% Nonidet P-40, 5% glycerol, 0.5 mM MgCl₂, 5 mM KCI, 0.05 mM EDTA, 3.5 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).

Electrophoretic Mobility Shift Assay (EMSA) —EMSA was carried out as described previously (9). Briefly, complementary DNA oligonucleotides were annealed by heating in 1× NET at 95°C for 5 min and cooling at ambient temperature. Probes were labeled with [γ-32P]ATP and T4 polynucleotide kinase. For gel shift assays nuclear extracts (6 μg) were incubated for 20 min at ambient temperature with 5×10⁴ 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, 0.7 μg/μl bovine serum albumin, 2 μg of poly(dI-dC). For supershift assays 2 μg of specific antibody was added and the reaction continued for 15 min. Samples were run on 6% nondenaturing polyacrylamide gels and electrophoresis carried out at 200 V in 25 mM Tris, pH 8.5, with 190 mM glycine and 1 mM EDTA. Competition assays were carried out in the same manner, except that the reaction mixtures were preincubated with competitor DNA for 10 min at 4°C before addition of the labeled probe. The relative binding avidities of various DNA probes to PU.1 were determined by comparisons of band intensities in the presence of a series of dilutions of competing constructs.

RESULTS

Functional Role of the Upstream Sequences Flanking the p47phox PU.1 Binding Site—Our previous work on the p47phox 5′ regulatory region showed that PU.1 is essential for promoter function (9). Using p47phox-based luciferase reporter gene constructs, we observed that the −46 construct containing the PU.1 binding site produced myeloid-specific expression, but that the −86 construct gave maximum promoter activity. Analysis of the p47phox −86 to −46 promoter region identified consensus sequences for Sp1 between positions −78 and −70 and for PEBP2 between positions −63 and −58 (9). However, gel shift experiments showed no evidence that either Sp1 or PEBP2 binds to these sites (data not shown). To investigate the role of the sequences immediately flanking the PU.1 site, we made a new construct pGL3-p47–48. In transient transfection assays of THP-1 cells (Fig. 1), pGL3-p47–48 exhibited twice the promoter activity of pGL3-p47–46, indicating the functional importance of the flanking nucleotides at positions −47 and/or −48 (T and C, respectively).

Effect of Flanking Sequences on PU.1-DNA Interaction in p47phox—We used EMSA to investigate the influence of the flanking nucleotides on PU.1 binding. To facilitate the direct correlation of DNA-protein binding with promoter activity, we designed DNA probes containing the PU.1 consensus motif flanked by the same sequences as those present in the corresponding reporter constructs used in transfections, including some vector sequences as required (Fig. 1A, under-
We observed several bands formed between the p47-PU.1–48 probe and THP-1 nuclear extracts (Fig. 2A, lane 2) and demonstrated binding specificity by competition with unlabeled DNA probe (Fig. 2A, lane 4). These bands contained PU.1 protein since the addition of PU.1-specific antibody resulted in major decreases in band intensity as well as the appearance of new supershifted bands (Fig. 2A, lane 3). Comparison with our previous studies (9) indicated that the major band contained intact PU.1 protein, whereas the faster-migrating bands were probably formed by PU.1 degradation products. The slower-migrating bands may contain other proteins in addition to PU.1. However, this appears unlikely because interaction of the p47-PU.1–48 DNA probe with in vitro synthesized PU.1 protein showed similar slow-migrating bands (Fig. 2D, lane 4), raising the possibility that these bands may contain PU.1 multimers. As will be discussed below, complex formation between the p47 DNA probe and in vitro synthesized PU.1 was readily competed by the unlabeled p47 probe, whereas only weak competition was observed with the CD18-PU.1 probe (Fig. 2D). The p47-PU.1–46 DNA probe exhibited

**FIG. 2. EMSA binding avidity analysis of PU.1 binding to p47-PU.1 DNA probes with or without the residues at positions –47 and –48.** Panel A demonstrates that PU.1 in THP-1 nuclear extract binds specifically to the p47phox promoter. 32P-Labeled p47-PU.1–48 DNA probe (see Fig. 1A) was incubated without (lane 1) or with (lane 2) THP-1 nuclear extract in the absence (lanes 1–3) or presence (lane 4) of excess unlabeled wild-type (Wt) probe. Where indicated (lane 3) 2 μg of antibody to PU.1 was added. DNA-protein complexes were separated on a 6% polyacrylamide gel. PU.1 > indicates the specific complex and SS > indicates the supershifted complex. Panels B and C are cross-competition studies showing that p47-PU.1 DNA probes bind to PU.1 more avidly if the residues at positions –47 and –48 are present than if they are absent. 32P-Labeled p47-PU.1–48 (panel B) or p47-PU.1–46 (panel C) DNA probes (see Fig. 1A) were incubated without (lane 1) or with (lanes 2–10) THP-1 nuclear extract in the absence (lanes 1 and 2) or presence of graded excesses of unlabeled DNA probe (lanes 3–10). Panel D shows a comparison of the binding of in vitro synthesized PU.1 protein to the p47phox promoter PU.1 site (p47-PU.1–48) versus the CD18 promoter distal PU.1 site (CD18-PU.1). 32P-p47-PU.1–48 was incubated either with THP-1 nuclear extract (lane 1) or with in vitro translated PU.1 (lanes 4–10) in the absence (lanes 1–4) or presence of graded excesses of unlabeled probe (lanes 5–10). Controls are shown for omission of nuclear extract (lane 2) and replacement of in vitro translated PU.1 by unprogrammed (i.e. no cDNA) reticulocyte lysate (lane 3; indicated by (−)).

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the same general binding patterns, as did p47-PU.1–48, including the slow-migrating bands (Fig. 2C, lane 2). However, based on cross-competition studies, PU.1 bound the p47-PU.1–48 probe twice as avidly as it did the p47-PU.1–46 probe (Fig. 2, B and C). These observations suggest that the nucleotides at positions −47 and/or −48 significantly influence PU.1 binding ability and thereby affect gene expression.

**Effects of Mutations at p47phox Position −48**—To test the influence of the nucleotide at position −48 on PU.1 transactivating activity, we mutated the wild-type nucleotide C to G, T, or A in the pGL3-p47–48 luciferase reporter construct (Fig. 3A). Transient transfection of THP-1 cells demonstrated comparable promoter activities of wild-type and each of the three −48-mutated constructs (Fig. 3B). Correspondingly altered oligonucleotides were studied by EMSA (Fig. 3C). These base substitutions had no effect on PU.1 binding, since each of the three mutated DNA probes was comparable to wild-type in its ability to compete with the labeled wild-type probe. Thus, the nucleotide at position −48 does not appear to influence either promoter activity or binding of PU.1.

**Effects of Mutations at p47phox Position −47**—Similarly, we mutated the wild-type nucleotide T at position −47 to A, C, or G (refer to Fig. 3A) and tested the effects on promoter activity and PU.1 binding. The mutant reporter constructs pGL3-p47–48-47A, pGL3-p47–48-47C, and pGL3-p47–48-47G exhibited only minimal promoter activity compared with the wild-type construct containing a T at position −47 (Fig. 4A). When one of these mutations was introduced into the larger construct pGL3-p47–86 to form pGL3-p47–86-47A, we again observed a
were similar to those in Fig. 2

The results of EMSA using wild-type and mutated DNA. Procedures

lanes 5–7

1

means (± S.E.) of at least four independent experiments. Panel B shows

were decreased by 50% and 85%, respectively, whereas those of

promoter.

p47phox

Effects of Mutations at Sites in the CD18 Promoter Analogous
to Positions −46 and −47 of p47phox—We hypothesized that

were G and T, respectively, exactly as for the comparable posi-
tions (−46 and −47) in the p47phox PU.1 flanking region.

A luciferase reporter construct, pGL3-CD18−81, containing the first 81 nucleotides upstream of the transcriptional start site was transiently expressed in THP-1 cells and compared with a construct (pGL3-CD18−81-76T77A) in which the flanking wild-type residues 76G and 77T were mutated to T and A, respectively (Fig. 6A). The promoter activity of the mutant construct was decreased by 75% relative to the wild-type (Fig. 6B). That this decrease was quantitatively similar to that seen with mutations of the PU.1 core sequence of CD18 (16) suggests that most of the contribution of the distal PU.1 binding site to the promoter activity of this construct was abrogated by mutation of the flanking residues. Next we investigated the distal PU.1 site of the CD18 promoter by EMSA, including comparisons with the p47phox PU.1 site (Fig. 6C). DNA probes CD18-

Panel B shows the results of EMSA using wild-type and mutated DNA. Procedures

were similar to those in Fig. 2A. 32P-Labeled p47-PU.1−48 probe (see

Fig. 1A) was incubated with THP-1 nuclear extract in the absence (lane

1) or presence of graded excesses of wild-type (46G, lanes 2–4) or

mutated (46C, lanes 5–7; 46A, lanes 8–10; 46T, lanes 11–13; 46T47A,
lanes 14–16) DNA. PU.1 > indicates the specific complex.

major decrease in promoter activity (Fig. 4A). When assessed by

EMSA, the oligonucleotides mutated at position −47 all failed to compete with the wild-type probe for PU.1 binding (Fig. 4B). Cross-competition studies suggested that the avidity of binding of the wild-type probe was about 3–9-fold greater than that of the mutants.

Effects of Mutations at p47phox Position −46—At position

−46, immediately adjacent to the core PU.1 consensus binding

sequence, the wild-type G residue was mutated to each of the

other three nucleotides (refer to Fig. 3A). In transfection studies

(Fig. 5A), promoter activities of the 46C and 46A mutants

were decreased by 50% and 85%, respectively, whereas those of

the 46T and combined 46T47A mutants were completely elimi-
nated. EMSA showed corresponding changes (Fig. 5B). Com-
pared with the wild-type 46G probe, the 46C mutant showed

moderately reduced binding, whereas the 46A, 46T, and

46T47A mutants each exhibited dramatically reduced binding.

The cross-competition studies suggested that the avidity of

binding of the wild-type probe was about 2–3-fold greater than

that of the 46C mutant and more than 9-fold greater than that

of the other three mutants tested.

Effects of Mutations at Sites in the CD18 Promoter Analogous
to Positions −46 and −47 of p47phox—We hypothesized that

our findings on the important functional roles of the nucleo-
tides flanking the p47phox PU.1 consensus binding site could be

extrapolated to other PU.1-regulated myeloid-specific genes.

To test this hypothesis, we chose the CD18 promoter as a

model. This promoter contains two PU.1 binding motifs: a
distal site at position −70 to −75 and a proximal site at posi-
tion −55 to −50. We focused on the distal site, which has been

shown to have functional activity that was dramatically de-

creased following mutation of the core PU.1 binding sequence

(16). Moreover, the flanking residues at positions −76 and −77

are G and T, respectively, as for the comparable posi-
tions (−46 and −47) in the p47phox PU.1 flanking region.

A luciferase reporter construct, pGL3-CD18−81, containing the first 81 nucleotides upstream of the transcriptional start site was transiently expressed in THP-1 cells and compared with a construct (pGL3-CD18−81-76T77A) in which the flanking wild-type residues 76G and 77T were mutated to T and A, respectively (Fig. 6A). The promoter activity of the mutant construct was decreased by 75% relative to the wild-type (Fig. 6B). That this decrease was quantitatively similar to that seen with mutations of the PU.1 core sequence of CD18 (16) suggests that most of the contribution of the distal PU.1 binding site to the promoter activity of this construct was abrogated by mutation of the flanking residues. Next we investigated the distal PU.1 site of the CD18 promoter by EMSA, including comparisons with the p47phox PU.1 site (Fig. 6C). DNA probes CD18-

PU.1 and p47-PU.1 formed similar patterns of complexes with

THP-1 nuclear extracts, but p47-PU.1 was far more active (by

about 20–30-fold) than CD18-PU.1 in the cross-competition

studies. The mutant probe CD18-PU.1−1−76,77A exhibited little

or no ability to bind PU.1 in THP-1 nuclear extracts (Fig. 6D).

When in vitro synthesized PU.1 protein was used instead of

THP-1 nuclear extract, similar bands were observed (see Fig.

2D), indicating the likelihood that PU.1 is the only nuclear

factor included in the complex. This experiment also confirmed

the greater ability of the p47-PU.1, compared with the CD18-

PU.1 probe to compete for PU.1 binding.

Correlation of Promoter Activity and PU.1 Binding Avidity—

Based on functional analyses of wild-type and mutated

p47phox reporter constructs and competition EMSA studies,

the relative promoter activities and PU.1 binding avidities

(estimated by cross-competition studies) were tabulated (Table

1). The Spearman rank correlation test showed a striking rela-
tionship between these parameters (r = −0.97, p < 0.0001).

DISCUSSION

We previously showed that the myeloid-specific transcription factor PU.1 is essential for the function of the p47phox gene promoter. In the present study, we demonstrate that the up-
stream nucleotides immediately flanking the PU.1 site in the

p47phox promoter (3′ to the GAGGAA sequence, since this is on

the non-coding strand) are important for full PU.1 binding and

functional activity. Mutations at base pair −46 or −47 dramat-
ically reduced the binding avidity and decreased or abolished

promoter activity, indicating the critical role of nucleotides G

and T, respectively, at these positions. The avidity of binding

to these sequences to PU.1 correlated very closely with their

ability to dictate reporter gene transcription. Analogous re-

results were obtained with the functional PU.1 site in the CD18

promoter.
gene promoter, suggesting a strong relationship between the avidity with which PU.1 binds its cognate sequence on a promoter and the resulting PU.1-mediated enhancer activity.

Based on the presence of potential binding sites, we speculated earlier (9) that the contribution to \( p47_{\text{phox}} \) promoter activity of the DNA sequence in the \(-286\) to \(-246\) interval might result from the binding of other transcription factors such as Sp1 and PEBP/CBP. Alternatively, increased activity might result from a PU.1-dependent effect of the nucleotides immediately flanking the consensus PU.1-binding site, GAGGAA. Our current work provides evidence for the latter mechanism by demonstrating that the nucleotides G and T at positions \(-246\) and \(-247\), respectively, are required for maximal PU.1 binding and \( p47_{\text{phox}} \) promoter activity. However, the contribution of other accessory factors cannot be completely excluded, since the transfection studies in THP-1 cells showed that the promoter activity of the \( pGL3-p47-48 \) construct was still only about half that of \( pGL3-p47-86 \), which we have shown previously to be sufficient for maximal promoter activity. In our previous studies, however, we also showed that mutation of the active PU.1 site from \( GA^GAA \) to \( CA^CCAA \) was equally effective in abolishing the promoter activity in \(-286\), \(-224\), and \(-2151\) \( pGL3-p47 \) constructs. Therefore, the function of any additional transcription factors regulating \( p47_{\text{phox}} \) transcription appears to be dependent on PU.1 binding.

This study shows that for the \( p47_{\text{phox}} \) promoter, the nucleotides G and T, which flank the 3′ end of the GAGGAA sequence, are the most active in promoting PU.1 binding and transactivation of the gene. Inspection of a number of functional PU.1 sites from other gene promoters (Table II) showed that these particular nucleotides occur most frequently at the corresponding positions in these other motifs (18G, 7C, 3A, and no T residues at positions analogous to \(-246\) and \(-247\), respectively, are required for maximal PU.1 binding and \( p47_{\text{phox}} \) promoter activity. However, the contribution of other accessory factors cannot be completely excluded, since the transfection studies in THP-1 cells showed that the promoter activity of the \( pGL3-p47-48 \) construct was still only about half that of \( pGL3-p47-86 \), which we have shown previously to be sufficient for maximal promoter activity. In our previous studies, however, we also showed that mutation of the active PU.1 site from GAGGAA to CACCAA was equally effective in abolishing the promoter activity in \(-86\), \(-224\), and \(-2151\) \( pGL3-p47 \) constructs. Therefore, the function of any additional transcription factors regulating \( p47_{\text{phox}} \) transcription appears to be dependent on PU.1 binding.

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**Table I**

| Construct     | Promoter activity (relative to basal level) | PU.1 binding avidity (rank order) |
|---------------|--------------------------------------------|-----------------------------------|
| pGL3-p47-48   | 13.9                                       | 1                                 |
| pGL3-p47-48A  | 14.4                                       | 2                                 |
| pGL3-p47-48G  | 13.9                                       | 3                                 |
| pGL3-p47-46C  | 7.3                                        | 5                                 |
| pGL3-p47-46   | 7.6                                        | 6                                 |
| pGL3-p47-47C  | 5.4                                        | 7                                 |
| pGL3-p47-47A  | 2.6                                        | 8                                 |
| pGL3-p47-47G  | 2.6                                        | 8                                 |
| pGL3-p47-46A  | 1.7                                        | 10                                |
| pGL3-p47-46T  | 0.8                                        | 11                                |
| pGL3-p47-48T47A | 0.9                                | 12                                |

\( ^{\text{a}} \) Spearman rank correlation test: \( r = 0.97; p < 0.0001. \)
flanking the PU.1 core consensus sequence are critical for full
PU.1 binding and transactivating activity.

Recent crystallographic studies of the PU.1 ets (DNA-bind-
ing) domain complexed with DNA also indicate the importance of the flanking sequences in binding of the PU.1 protein (6). In these studies, the PU.1 ets domain was shown to bind the DNA with novel loop-helix-loop architecture and to form a series of contacts with both the core GGAAG sequence and with nucleo-
tides 3' to this motif. A number of amino acid residues contact the phosphate backbone of the two nucleotides immediately 3' to the GGAAG sequence (corresponding to positions −46 and −47 of the p47phox PU.1 site). Substitution of some of these amino acids with glycine was sufficient to abolish binding to the DNA (6). Similarly, nucleotides immediately 5' to the GAG-
GAA sequence were shown to be contacted by amino acid res-
ides of the ets domain and likewise to be important in PU.1-
DNA interaction (5). In agreement with these latter data, we have found that specific nucleotides 5' to the PU.1 core se-
quence in the p47phox promoter are also required for full PU.1 binding and transactivation of the gene.2

Paxton and colleagues (25) demonstrated that specific se-
quences flanking a site for NF-xB are required for cognate transcription factor binding and tumor necrosis factor-α-mediated induction of intercellular adhesion molecule-1. Mutations of either the 5'- or 3'-flanking regions abrogated tumor necrosis
factor-α-induced reporter activity, as did mutations of the core
NF-xB site. A specific DNA-protein complex was formed when wild-type flanking sequences were included in the EMSA
probe, but no complex was formed when random flanking se-
quences were used. Whether there was a quantitative correla-
tion between binding affinity and promoter activity was not
directly addressed. To our knowledge, the current paper is the
first to provide a systematic quantitative correlation between
transcription factor binding avidity and promoter activity of
specific nucleotide sequences. As shown in Table I, flanking
sequences determine the avidity of PU.1 binding and thereby
influence p47phox promoter activity. Nevertheless, the corre-
lation appears to be promoter-dependent. For example, com-
paring the p47phox promoter and the CD18 promoter, the
wild-type PU.1 binding site of the p47phox gene binds to PU.1
30-fold more strongly than does the wild-type CD18 PU.1 site.
If the p47phox PU.1 site is mutated to have such a low binding
affinity, it completely loses its contribution to promoter
activity.

PIP has been reported to bind to the specific flanking nucleo-
tides of PU.1 sites in the presence of bound PU.1 and thereby
increase promoter activity (7). On the other hand, FEF-1 binds
to the adjacent sequences independently of PU.1, but cooper-
ates with PU.1 to enhance gene transcription (8). Our current
findings that the flanking sequences of PU.1 binding sites
influence promoter activity and gene expression by altering the
binding avidity of PU.1 provide another model for the mecha-
nism by which the flanking sequences affect transcription. We
propose that such a mechanism may also operate in other cis-
element-transcription factor interactions.

The phagocyte NADPH oxidase is an enzyme complex com-
prised of several protein subunits, principally the membrane
components gp91phox and p22phox and the cytosolic components
p67phox, p47phox, and Rac1/2 (19, 20). Genetically determined
deficit of NADPH oxidase activity results from mutations in the
genes for gp91phox or p47phox or, rarely, the genes for p22phox or
p67phox, and leads to the clinical disorder CGD (21, 22). Most
cases result from mutations in the coding sequence of the af-
ected gene. However, variant forms of gp91phox-deficient
CGD have been described in which low levels of the protein are
expressed and single base changes are found at positions −52,
−53, −55, or −57 of the active PU.1 site of the gp91phox
promoter (10, 26, 27). The consensus PU.1 motif GAGGA of the
gp91phox gene promoter is located between −50 to −55
relative to the transcription start site. Position −57 corre-
sponds to position −47 of the p47phox promoter, shown in the
current work to be critical for PU.1 binding and promoter
activity. In vitro mimicking of the CGD sequences by mutation of
the PU.1 core motif of the gp91phox promoter from GAGGA to
GAGGAG or the flanking sequence from GAGGAAAT to
GAGGAG, and leads to the clinical disorder CGD (21, 22).
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sponds to position −47 of the p47phox promoter, shown in the
current work to be critical for PU.1 binding and promoter
activity. In vitro mimicking of the CGD sequences by mutation of
the PU.1 core motif of the gp91phox promoter from GAGGA to
GAGGAG or the flanking sequence from GAGGAAAT to
GAGGAAG to GAGGAG led to major losses in promoter activity (10).
In the case of p47phox-deficient CGD patients, a single lesion,
A GT deletion at an intron-exon junction is the predominant
mutation. The recent identification of a p47phox pseudogene
that contains the GT deletion but is otherwise highly homolo-
gous to the normal gene suggests that recombination events,
for example crossovers with deletions and/or gene conversions,
between the p47phox gene and the pseudogene account for
most cases of p47phox deficiency (28). To date, no CGD patients
with mutations in the p47phox promoter have been described.
In part, this may be due to the rarity of this genetic disorder.
Second, it could be a consequence of activation of the p47phox
gene by regulatory elements outside of the promoter
region that we have studied. Third, coding regions tend to comprise
longer stretches of DNA and therefore are more likely to
manifest mutations than are promoter elements, which are usually
clustered in small regions upstream of coding segments.
Fourth, redundancy of promoter elements may allow for at
least partial compensation for mutations of core or flanking
sequences of a promoter motif, thereby resulting in subtle
phenotypes that could be overlooked clinically. Thus, it re-
mains to be seen whether mutations of p47phox PU.1 core or
flanking binding sequences may account for some clinical forms
of CGD, as is the case for the gp91phox gene.

| Table II: Survey of nucleotide sequences of functional PU.1 binding sites |
|-----------------------------|-----------------------------|
| Promoter or enhancer | Core and flanking nucleotide sequence |
| p47phox               | GAAGAAGT |
| gp91phox              | GAAGAAGT |
| CD18 (distal)         | GAAGAAGT |
| CD18 (proximal)       | GAAGAAGA |
| CD11b                 | GAAGAAGT |
| CD20                  | CAAAGAGT |
| CD33                  | GAAGAAC |
| M-CSFR                | GGGAAGA |
| G-CSFR                | GGGAAGC |
| GM-CSFR               | GGGAAGC |
| FcγRI                 | GGGAAGG |
| FcγRIIIA              | GGGAAGG |
| Mannose receptor      | CAAAGAG |
| IL-1β (distal)        | GGGAAGG |
| IL-1β (proximal)      | CAAAGAG |
| IL-4                  | CAAAGAC |
| sIL-1 receptor antag. | GGGAAGAT |
| Igκ 3'                | GAAAGCT |
| Igλ 2–4               | GAAAGCT |
| Igμ                   | GGGAAGG |
| Igδ                   | CAAAGGC |
| Igκ Vκ19              | GAAAGGT |
| Neutrophil elastase   | GAAGAGT |
| Chicken lysozyme      | CAAAGCT |
| Scavenger receptor    | CAAAGCT |
| SV40                  | CAAAGCG |
| Equine infectious anemia virus long terminal repeat | CAAAGGT |
| Epstein-Barr virus latent membrane protein | GGGAAGT |

* References for promoter sequences (9, 10, 16, 29–36).
* Nucleotides flanking core sequence shown in bold font.

2 S.-L. Li, A. J. Valente, and R. A. Clark, unpublished observations.
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