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Differentiation-dependent up-regulation of p47^{phox} gene transcription is associated with changes in PU.1 phosphorylation and increased binding affinity

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Abstract

The p47^{phox} gene encodes a cytosolic component of the phagocytic NADPH oxidase complex. Expression of p47^{phox} is both tissue-specific and developmentally regulated. Stable transfection of the myeloid cell lines PLB985 and HL60, with reporter gene constructs containing as little as 58 bp of proximal promoter sequence, was capable of directing significant reporter gene activity in myeloid cells, which increased significantly on induction of myeloid differentiation. EMSA analysis of a binding site for the *Ets* family member, PU.1, located at positions −39 to −44 revealed that the pattern of complex formation changed significantly on induction of myeloid differentiation. All EMSA complexes were competed by a functional PU.1 binding site and could be supershifted in the presence of polyclonal anti-PU.1 antibody. Reaction of EMSA complexes with anti-phosphoserine antibody, treatment with phosphatase, or Western blotting of proteins captured on the PU.1 binding site, was used to demonstrate that the changes in PU.1 complex formation dependent on myeloid differentiation were associated with increased levels of PU.1 phosphorylation. Furthermore, the more highly phosphorylated forms of PU.1 were shown to have a greater affinity for the p47^{phox} PU.1 consensus binding site. Up-regulated transcriptional activity in response to myeloid differentiation can therefore be correlated with increased levels of PU.1 phosphorylation and a greater binding affinity.

Analysis of many myeloid-specific gene promoters has indicated that a common determinant of myeloid gene expression in terminally differentiated cells is the myeloid and B cell-specific transcription factor PU.1 [1–3]. A functional binding site for PU.1 is located in close proximity to the transcriptional start site of numerous myeloid-specific genes, including the macrophage colony stimulating factor receptor (M-CSFR) gene, *c-fms*, FcRγI, CD11b, and gp91^{phox} [3]. The promoter of the gene encoding p47^{phox}, a component of the NADPH oxidase of phagocytes, has a consensus PU.1 binding sequence GAGGAA [4] located in anti-sense orientation between positions −39 and −44 of the promoter. PU.1 binding at this site, which is required for reporter gene expression in transient assay, has been reported in HL60 cell nuclear extracts [5]. In common with many myeloid-specific genes [3], p47^{phox} is markedly up-regulated on induction of terminal myeloid differentiation [6], with steady-state levels of mRNA increasing over 50-fold [7,8]. However, little is currently known about the mechanism of this transcriptional up-regulation.

In this report, we show that a promoter consisting of only the proximal 58 bp of 5′ p47^{phox} flanking sequence is sufficient to direct transcription in stably transfected myeloid cells. In stable transfectants, in contrast to the results obtained by transient assay, this minimal promoter was also found to direct significant up-regulation

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of reporter gene transcription in response to induced myeloid differentiation. Moreover, up-regulation of transcription from the p47^{phox} promoter could be correlated with changes in PU.1 binding complexes, which were shown to be due to increased levels of PU.1 phosphorylation. These hyperphosphorylated forms of PU.1 also showed evidence of increased affinity for the p47^{phox} PU.1 consensus binding sequence.

Materials and methods

Materials and cell cultures. Chemical reagents were purchased from Sigma (Sigma–Aldrich, Dorset, UK) unless otherwise stated. Molecular biological reagents, including restriction and modification enzymes, were purchased from Promega (Promega Corporation, Southampton, UK), unless otherwise stated. Rabbit polyclonal immunoglobulin (Ig) G anti-PU.1 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Santa Cruz, CA). Oligonucleotides were purchased from Sigma–Genosys Biotechnologies (Europe), (Cambridge, UK). Oligonucleotides used as PCR primers in the construction and detection of p47^{phox}CAT*neo* plasmids were as follows, with restriction enzyme sites underlined and named in brackets: −58 to −45 p47^{phox} promoter, 5′-TGCAAGCTTGCGACA AAAGCGAC-3′, (*Hin*dIII); +22 to +5 p47^{phox} sequence, 5′-CGCGTCTAGACTGGGTGGCCTCCAGT-3′, (*Xba*I); 2215 to 2233 pCAT-Basic plasmid (Promega), Those used for competition experiments were: −104 to −84 of FcγRIb promoter containing the PIE motif [9] [PIE] 5′-GCAATTTCCTTCCTCTTTTCT-3′; −68 to −30 gp91^{phox} promoter [10] [HAF] CTGCTGTTTCATTTCCTCATTGGA AGAAGAAGCATTGT; −62 to −35 of human class II HLA-DRα promoter [11] [OCT], 5′-AGAGTAATTGATTTGCATTTTAA TGGTC-3′. All cell lines used in this study were of human origin. The promyelocytic leukemic cell line HL-60, the B lymphoblastoid cell line Daudi, the myelomonocytic cell line U937, and the hepatocyte carcinoma cell line HepG2 were obtained from the European Collection of Cell Cultures (ECCC, Salisbury, UK). The human diploid myeloid leukemia cell line PLB985 was a generous gift of Dr. Mary Dinauer, Indiana University Medical Centre, Indianapolis, IN, USA. All cell lines were grown at 37 °C and 5% CO_2. Myeloid and lymphoblastic cell lines were cultured in RPMI 1640 (Life Technologies, Paisley, UK) supplemented to 10% heat-inactivated fetal bovine serum (HI-FBS), 2 mM L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin, with the exception of the Daudi cell line, which was cultured in the presence of 80 μg/ml gentamycin (Roussel Laboratories, Middx, UK) as antibiotic. Non-haematopoietic cell lines were cultured in Dulbecco's Modified Eagle's Medium (Gibco-BRL) supplemented to 10% HI-FBS, 2 mM L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. Myeloid cell lines were induced to differentiate to a granulocytic phenotype by addition of 1.25% DMSO or monocyte/macrophage phenotype with 40 nM phorbol myristate acetate (PMA) (Sigma). Differentiation was confirmed by nitroblue tetrazolium (NBT) staining [12]. Multiple independent transfectants were analyzed.

Plasmid construction. The Δ58p47^{phox}CATneo promoter construct was produced by insertion of a p47^{phox} promoter PCR fragment (reverse primer, +22 to +5 p47^{phox} sequence; forward primer −58 to −45 p47^{phox} promoter, respectively) directionally cloned into *Hin*dIII and *Xba*I sites located directly upstream of the promoterless CAT gene in the pCAT-Basic plasmid (Promega) into pCAT-Basic. Construct identity was confirmed by restriction mapping and sequencing. To allow the selection of stable transfectants, an SV40-driven neomycin phosphotransferase expression cassette was inserted as a *Bam*HI fragment into *Bam*HI-linearised p47^{phox} promoter reporter constructs to produce Δ58p47^{phox}CATneo.

Stable transfection. The hematopoietic cell lines HL60 and PLB985 were transfected by electroporation following a modification of the method of Pahl et al. [13]. Cells growing in log phase were resuspended in conditioned media at a density of 4×10^7 cells/ml. Fifty micrograms of uncut reporter gene plasmid DNA was resuspended in a volume of TE of not more than 5% of the total cell volume, mixed with 250 μl cells in 0.4 cm electrode gap electroporation cuvettes (Bio-Rad Laboratories, Herts, UK), and incubated for 10 min at room temperature. Cells were electroporated using a Bio-Rad Gene Pulser II at 950 μF, with voltage as follows: HL60, 300 V; PLB985, 275 V. Cells were incubated at room temperature for 20 min (PLB985), or on ice for 15 min and transferred to 10 ml RPMI 1640 supplemented as previously described plus 20% re-conditioned media. Stably transfected cells were incubated at 37 °C, 5% CO_2. Selection in 0.5 mg/ml G418 was begun 24–72 h after transfection and continued for 5–6 weeks. Cultures were maintained as polyclonal; individual transfectants were not cloned out and multiple independent cultures were established.

Reporter gene assay. Assay of CAT enzymatic activity was performed by Thin Layer Chromatography (TLC). Cells were washed three times in phosphate-buffered saline (PBS, Oxoid Unipath , Basingstoke, UK) and lysed in 100 μl of 0.25 M Tris–HCl, pH 7.8, by three freeze–thaw cycles of 5 min incubation in liquid nitrogen followed by 5 min incubation at 37 °C. The lysate was cleared twice by centrifugation at 15,000*g*, 4 °C for 10 min. Soluble protein content was determined by Bio-Rad Protein Assay (Bio-Rad Laboratories). Extract containing 100–200 μg protein in 104 μl of 0.25 M Tris–HCl, pH 7.8, was incubated at 60 °C for 10 min, ice-chilled and centrifuged at 15,000*g* for 30 s. Extract was incubated at 37 °C for 20 h in the presence of 0.55 mM acetyl CoA and 0.0575 μCi [^{14}C]chloramphenicol (Amersham Life Science, 25 μCi/ml) in a total volume of 168.7 μl. Extract was centrifuged at 2100*g* for 30 s and the reaction terminated by the addition of 1 ml ethyl acetate (BDH-Merck, Lutterworth, UK). The sample was vortexed for 30 s and centrifuged at 15,000*g* for 2 min. One ml of the organic phase was transferred to a fresh tube and vacuum-dried for 45 min. The residue was resuspended in 20 μl ethyl acetate and loaded onto a plastic silica gel TLC plate (BDH-Merck) 1.7 cm from the bottom at 1.5 cm horizontal intervals. Chromatography proceeded for 13 min in 200 ml of 95% chloroform, 10% methanol (pre-equilibrated for 1 h). The air-dried silica plate was subjected to autoradiography with Hyperfilm β-max (Amersham Life Science) for 24 h at room temperature. CAT activity was determined in lysates containing equal amounts of protein in order to compensate for the decrease in cellular protein that occurs during myeloid differentiation.

In vitro binding assays. Soluble nuclear extracts were prepared from 5×10^8–2×10^9 cells growing in log phase, by modification of the method of Dignam et al. [14]. Briefly, cells were resuspended in break buffer (0.3 M sucrose, 10 mM Hepes–KOH, pH 7.9, 10 mM KCl, 0.1 mM EGTA, and 1.5 mM $MgCl_2$) containing freshly added 0.01 mM leupeptin, 0.01 mM pepstatin, 0.5 mM phenylmethonylsulphonyl fluorophosphate (PMSF), and 0.5 mM dithiothreitol (DTT); NP40 was added to 0.4% for cell lysis. Pelleted nuclei were washed twice in break buffer without NP40, re-pelleted and homogenized in buffer containing 10 mM Hepes–KOH, pH 7.9, 400 mM NaCl, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 5% v/v glycerol plus freshly added 0.5 mM DTT, and 0.5 mM PMSF. Homogenate was slowly stirred at 4 °C for 30 min and centrifuged at 100,000*g*, 4 °C for 60 min to remove insoluble material. Soluble proteins were dialyzed at 4 °C for 2–4 h against 50 vol of dialysis buffer (20 mM Hepes–KOH, pH 7.9, 75 mM NaCl, 0.1 mM EDTA, 20% v/v glycerol, 0.5 mM DTT, and 0.5 mM PMSF). The extract was cleared by centrifugation at 25,000*g*, 4 °C for 15 min. Soluble protein content was determined as described above. Double-stranded oligonucleotides used as ^{32}P-labelled EMSA probe or unlabelled competitor were constructed by annealing and filling out of complementary oligonucleotide pairs, designed to overlap by 8–10 nucleotides at their center. Annealing was performed at a concentration of 5 μM per oligo in a total volume of 50 μl in 1× Klenow buffer (50 mM Tris–HCl, pH 7.6, 10 mM $MgCl_2$) by incubation at 70 °C for

1 min, slow cooling to 25 °C at a rate of 9 s/°C, and incubation at 25 °C for 1 min. The unpaired regions of annealed oligonucleotide pairs were filled out (25 pmol) by incubation in 1× Klenow buffer in the presence of 0.1 mM dATP, dCTP, dGTP, TTP (Promega), and 5U DNA Polymerase I large (Klenow) fragment in a total volume of 20 μl for 30 min at room temperature. Unincorporated nucleotides were removed by separation through Sephadex G50. Annealed complementary oligonucleotide pairs (25 pmol) and DNA restriction fragments (50 ng) used as EMSA probes were ^{32}P end-labelled by incubation in the presence of 1× Klenow buffer, 0.1 mM dATP, dGTP, dTTP (Promega), 50 μCi [α-^{32}P]dCTP (Amersham Life Science, 3000 μCi/mmol), and 5U DNA Polymerase I Klenow fragment (Promega) in a total volume of 20 μl at room temperature for 20 min. The reaction was chased by the addition of unlabelled dCTP to 0.05 mM and further incubation at room temperature for 5 min. Unincorporated nucleotides were removed as described previously.

Electrophoretic mobility shift assay. Electrophoretic mobility shift assay (EMSA) [15] binding reactions were performed in a total volume of 30–50 μl. The binding of nuclear protein (3 μg) to 1 ng ^{32}P-labelled DNA restriction fragment probe or 0.25 pmol oligonucleotide probe was performed in binding buffer (10 mM Tris–HCl, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 12.5% v/v glycerol, 0.1% Triton X-100, and 1 mM DTT, freshly added) for 30 min at room temperature or 4 °C, respectively, in the presence of a minimum of 41.7 μg/ml unlabelled non-specific competitor DNA [polydeoxyinosinic–deoxycytidylic acid (poly(dI–dC))]. A poly(dI–dC) titration was initially performed for each cell type in order to ensure specific complex formation; complex formation in myeloid cells remained stable at 333.3 μg/ml poly(dI–dC). Competition experiments with unlabelled double-stranded oligonucleotide were performed by pre-incubation of the binding reaction either at room temperature (restriction fragment probe) or 4 °C (oligonucleotide probe) for 15 min, in the absence of labelled DNA. DNA–protein complexes were electrophoretically separated from unbound probe on 4–6% polyacrylamide in 1× gel running buffer (1 mM EDTA, 6.7 mM Tris–HCl, pH 7.5, 3.3 mM NaOAc, continuously circulated by peristaltic pump) at room temperature (restriction fragment probe) or 4 °C (oligonucleotide probe). Gels were dried under vacuum onto Whatman 3MM paper (Whatman International, Maidstone, UK), those using oligonucleotide probes being fixed in 10% methanol, 10% acetic acid (oligonucleotide probe) prior to drying. Autoradiography was performed at −70 °C for 14–16 h in the presence of intensifying screens. Antibody supershift experiments were performed by addition of 2 μg of antibody prior to, or following, the binding reaction and further incubation at 4 °C for 1 h. Anti-PU.1 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-phosphoserine monoclonals were purchased from Affiniti Research (Exeter, UK). On some occasions the binding pattern obtained was compared to that obtained when nuclear extract was prepared in the presence of phosphatase inhibitors (Sigma, Poole, UK). No differences in the protein binding patterns were observed in the presence of phosphatase inhibitors.

UV cross-linking. EMSA complexes, as described above, were UV cross-linked at 254 nm for 20 min at room temperature using a CL-100 cross-linker (UVP, Upland, CA). The cross-linked nuclear protein extracts were separated on 12.5% SDS–PAGE gels as described below for Western blotting.

Phosphatase treatment. EMSA binding reactions were treated with calf intestinal alkaline phosphatase (Boehringer–Mannheim, Sussex, UK) for 30 min at 37 °C or potato acid phosphatase (Sigma–Aldrich) at 30 °C for 30 min.

Affinity isolation of PU.1 and Western blotting. PU.1 was isolated from HL60 nuclear extract using a 5′ biotinylated P oligonucleotide probe in a binding reaction, as described for EMSA, but scaled up 25 times. The oligonucleotide was captured (15 min at 4 °C) onto 50 μl of magnetic beads using the μMacs system (Miltenyi Biotech, Surrey, UK). The proteins bound to the oligonucleotide were eluted with 1× SDS–PAGE sample buffer pre-warmed to 95 °C. Total nuclear extract proteins (2 μg) or material captured on 5′ biotinylated P oligonucleotide were separated on 12.5% SDS–PAGE and electro-blotted onto reinforced nitrocellulose membrane (Schleicher & Schuell BA-S 85) for 1 h at 5.5 mA/cm^2 by semi-dry blotting (Bio-Rad Trans-Blot SD) in Tris–glycine running buffer supplemented with 20% methanol. Transfer was monitored by the use of pre-stained standards (Sigma–Aldrich) and staining with 2% Ponceau-S (Sigma–Aldrich). Blots were blocked for 16–48 h at 4 °C in TBST (20 mM Tris–HCl, pH 7.6, 0.15 M NaCl, and 2% Tween 20) supplemented with 3% BSA and 5% fetal calf serum and reacted with primary antibody (1:1000) for a further 16–24 h at 4 °C. The membranes were then washed five times (5 min each) in TBST and reacted with a 1:2000 dilution of peroxidase conjugated secondary antibody (anti-rabbit IgG or goat anti-mouse IgM) in TBST for 1 h at 20 °C, washed in TBST, as before. The blots were developed using femtoLucent (Chemicon, Temecula, CA) chemiluminescent detection and exposure (0.5–5 min) to X-ray film (Hyperfilm MP, Amersham Pharmacia Biotech, Bucks, UK).

Results

58 bp of p47^{phox} promoter sequence is sufficient to direct reporter gene expression in myeloid cells and is up-regulated in response to myeloid differentiation

Analysis of CAT reporter plasmids containing varying amounts of 5′ flanking sequence (CMM and CC unpublished obs.) allowed us to determine a minimal functional p47^{phox} promoter. A construct (Δ58p47^{phox} CAT*neo*) containing as little as 58 bp of proximal promoter sequence (Fig. 1A) was found to be sufficient to direct significant levels of reporter gene activity in stably transfected HL60 myeloid cells (Fig. 1B, lanes H1 and H2).

More significant however, was a distinct increase in transcription observed in response to DMSO-induced myeloid differentiation (Fig. 1C, PLB985, lanes 2–4, 5–7; HL60, lanes 8–10). This was established for three independently isolated clones in PLB985 cells and in five out of six independent clones in HL60. The sixth HL60 clone was found to be inactive, due to the effects of integration site on expression. The data are summarized in Fig. 1D. The increases at Day 5 were significant at $p < 0.05$ for the individual cell lines and at $p < 0.01$ when the data from both lines were combined (Student's *t* test). The increases were more marked in the PL985 cells (about 3.5-fold) owing to a generally lower Day 0 level of transcription.

Complex formed on the 58 bp proximal promoter localizes to the PU.1 consensus binding site

We then investigated protein binding to this minimal p47^{phox} promoter region to establish whether changes in the pattern of transcription factor binding were associated with the differentiation-dependent up-regulation of transcription we observed.

EMSA was performed using a ^{32}P-labelled *Xba*I/*Hin*dIII DNA restriction fragment probe containing 58 bp of

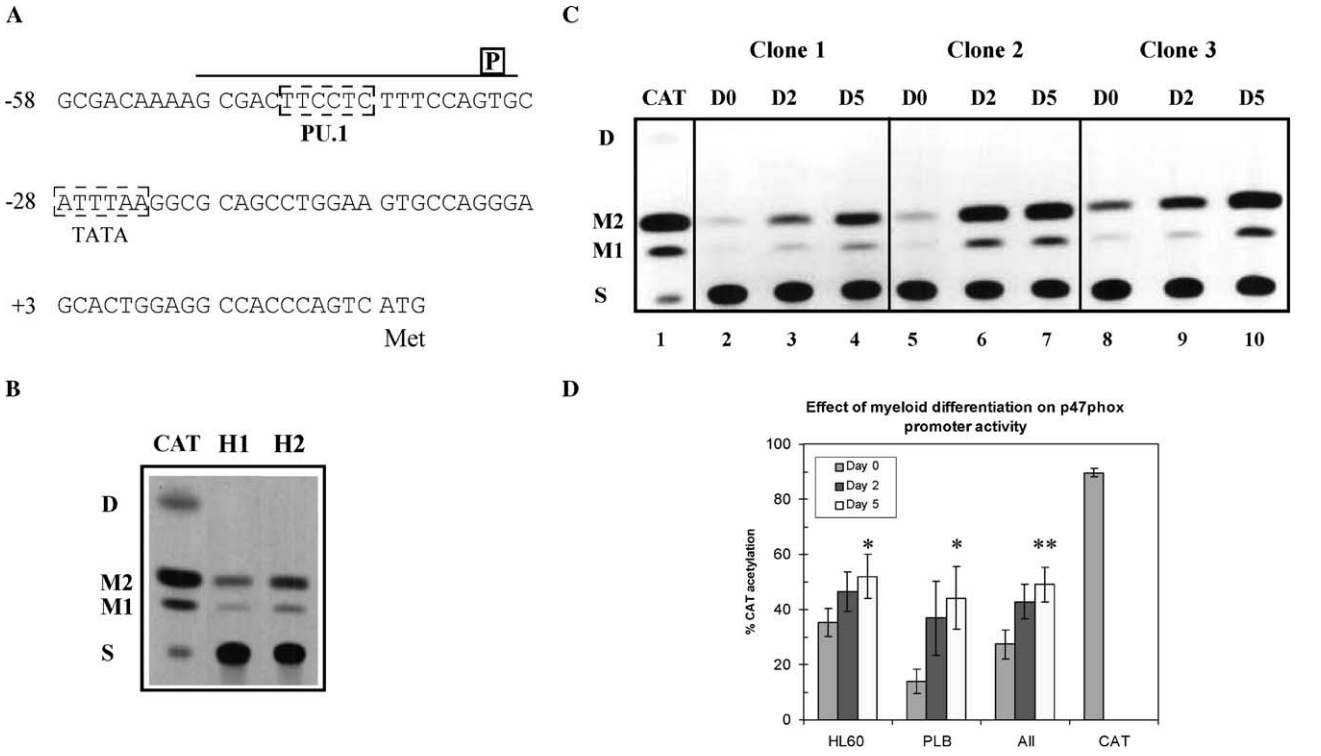

Fig. 1. The proximal 58 bp of the p47^{phox} promoter is sufficient to direct transcription in myeloid cells and up-regulates in response to myeloid differentiation. (A) p47^{phox} promoter sequence. The first 58 nucleotides upstream of the start site of transcription is shown. The sequence is numbered according to the sequence of Thrasher (A.J. Thrasher, Ph.D. Thesis, 1995, University of London, UK; GenBank Accession No. U33006), with the transcriptional start site (TSS) designated as position +1 as determined by Rodaway et al. [6]. The hatched box indicates the position of a functional PU.1 binding motif investigated in this report. Oligonucleotide probe P used for EMSA is indicated by overlined sequence. The translational initiation site (Met) is indicated below the sequence. (B) The proximal 58 bp of the p47^{phox} promoter directs CAT reporter gene expression. CAT activity was determined in lysate from two independently isolated HL60 clones (H1 and H2) stably transfected with the pCAT-58neo construct. Activity from 0.5 U CAT enzyme (CAT) is shown for comparison. (C) The proximal 58 bp of the p47^{phox} promoter up-regulates in response to myeloid differentiation. CAT activity was determined in PLB985 (lanes 2–7) and HL60 (lanes 8–10) myeloid cell lines stably transfected with the pCAT-58neo construct. Transfectants were differentiated to a granulocytic phenotype in the presence of DMSO. CAT activity was determined in 100 μg cellular lysates from undifferentiated transfectants (D0/lanes 2, 5, and 8) or from transfectants treated for 48 h (D2/lanes 3, 6, and 9) or 120 h (D5/lanes 4, 7, and 10), or activity of 0.5 U CAT enzyme alone (lane 1). D, M1, M2, and S identify the migration of diacetylated, two monoacetylated, and unacetylated forms of chloramphenicol, respectively. (D) Histograms showing mean (and SEM) increases in p47^{phox} promoter activity on DMSO-induced differentiation of HL60 ($n = 5$) and PLB 985 ($n = 3$) myeloid cell transfectants, and pooled data (all). The acetylase activity of 0.5 units of pure CAT enzyme is shown for comparison. Asterisks indicate statistically significant difference between this time point and time zero (paired t test) *, $p < 0.05$; **, $p < 0.01$.

proximal p47^{phox} promoter sequence (Fig. 2A). A single complex (C1) was formed on this probe in nuclear extract from undifferentiated PLB985 cells (Fig. 2A, lane 2). To characterize the C1 complex binding site, competition experiments were performed with unlabelled double-stranded p47^{phox} promoter oligonucleotides. Complex formation was completely abolished in the presence of 50-fold molar excess of a p47^{phox} cognate oligonucleotide [P] comprising −53 to −29 bp of p47^{phox} promoter sequence (Fig. 2A, lane 3), but not by a 100-fold excess of a P oligonucleotide containing a mutated consensus PU.1 binding site [Pmt] (Fig. 2A, lanes 5 and 6).

The binding specificity of this complex was investigated by competition experiments performed with oligonucleotide competitors containing functional protein binding motifs. C1 complex formation was significantly disrupted by 50- or 100-fold molar excess of oligonucleotides containing functional PU.1 binding sites: the PIE region of the myeloid-restricted high affinity Ig receptor FcRγIb gene promoter [PIE] [9] (Fig. 2A, lanes 9 and 10) and to a lesser extent by the HAF1 site of the gp91^{phox} gene promoter [10,16] (lanes 7 and 8). Formation of complex C1 was not disrupted by at least 100-fold excess of a B-cell specific octamer binding site [OCT] [11] (Fig. 2A, lane 12).

The protein binding studies strongly implicated the consensus binding site for the myeloid and B-cell-specific transcription factor PU.1 [4], located in antisense orientation between positions −39 and −44 (Fig. 1A), as the site of C1 complex formation. We have demonstrated that mutation of this site in the context of a larger construct knocks out reporter gene expression in stable transfectants (CMM and CMC unpublished obs.). Further EMSA experiments were, therefore, per-

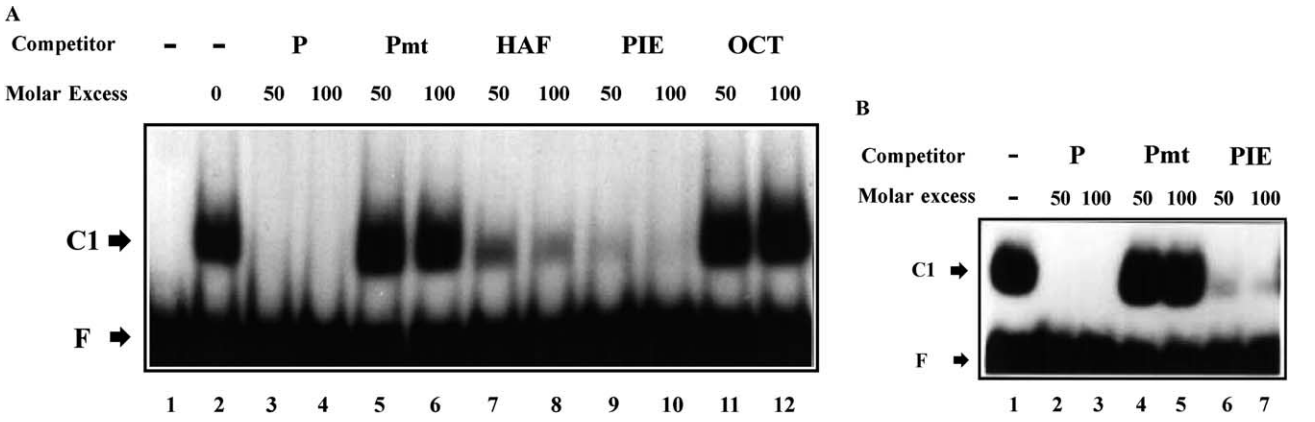

Fig. 2. Electrophoretic mobility shift analysis (EMSA) of the proximal 58 bp of the p47^{phox} promoter. (A) EMSA was performed with a ^{32}P-labelled double stranded DNA restriction fragment probe containing 58 bp of the p47^{phox} proximal promoter (lanes 1–12) and 3 μg nuclear extract from undifferentiated PLB985 cells (lanes 2–12), or in the absence of extract (lane 1). Specific complex formation is indicated by C1; free probe is indicated by F. Competition experiments were performed using 50-fold (lanes 3, 5, 7, 9, and 11) or 100-fold (lanes 4, 6, 8, 10, and 12) molar excess of double stranded unlabelled oligonucleotide as indicated. Oligonucleotide competitors were as follows: lanes 1 and 2, none; lanes 3 and 4, p47^{phox} promoter PU.1 consensus binding site (P); lanes 5 and 6, oligonucleotide P with mutated PU.1 consensus binding site [Pmt]; lanes 7 and 8, gp91^{phox} promoter HAF binding site (HAF) containing the Ets family binding consensus; lanes 9 and 10, FcγRI promoter (−104 to −84) containing PU.1 binding site [PIE]; lanes 11 and 12, human class II HLA-DRα promoter (−65 to −35) containing a B-cell specific octamer binding site (OCT). (B) EMSA was performed as described in Fig. 3A, with ^{32}P-labelled oligonucleotide probe P and undifferentiated PLB985 cell nuclear extract. Specific complex formation is indicated by C1; free probe is indicated by F. Competition was performed using 50-fold (lanes 2, 4, and 6) or 100-fold (lanes 3, 5, and 7) molar excess of unlabelled oligonucleotide as indicated. Competitors are as follows: lane 1, none; lanes 2 and 3, p47^{phox} promoter PU.1 consensus binding site oligonucleotide [P]; lanes 4 and 5, oligonucleotide P with mutated PU.1 consensus binding site [Pmt]; lanes 6 and 7, FcγRI PU.1 binding site [PIE].

formed using the p47^{phox} promoter oligonucleotide P (see Fig. 1A) as probe (Fig. 2B). A single complex was formed in undifferentiated PLB985 nuclear extracts (Fig. 2B, lane 1), in agreement with previous EMSA data (Fig. 2A, lane 2). Significantly, no complex formation was observed using the p47^{phox} oligonucleotide Pmt (containing a mutated PU.1 binding site) as probe (data not shown). The pattern of competition of the oligonucleotide P complex was identical to that of C1 complex formation at the −58 DNA restriction fragment probe (see Fig. 2A). Complex formation was abolished by 50- and 100-fold molar excess of oligonucleotide P (Fig. 2B, lanes 2 and 3), or the FcRγIb promoter PU.1 binding site [PIE] (Fig. 2B, lanes 6 and 7) and the gp91^{phox} promoter site [HAF] (data not shown) but not by oligonucleotide Pmt (Fig. 2B, lanes 4 and 5) or an OCT oligonucleotide, containing a B-cell-specific octamer binding motif (data not shown). These results indicated that complex formation was restricted to the PU.1 binding site and that complex C1 contained PU.1, or a closely related factor.

Complexes formed at the PU.1 consensus binding site alter significantly in response to myeloid differentiation

To determine whether differentiation-related changes in transcription factor binding could be visualized, complex formation was investigated in a number of myeloid cell types, before and after induced differentiation (Fig. 3A, lanes 2–7), and in the Daudi B-cell line (Fig. 3A, lane 1) which has constitutive high level p47^{phox} transcription. In undifferentiated cells, in contrast to the single high-mobility complex C1 formed in nuclear extract from PLB985 cells (Fig. 2B, lane 1, Fig. 3A, lane 2), three new complexes were identified. In U937 cells two additional complexes of lower mobility, C2 and C3, were present (Fig. 3A, lane 6) and in HL60 cells, a further lower-mobility complex, C4 was formed (Fig. 3A, lane 4). With nuclear extract from Daudi B-cells, which have constitutive high-level p47^{phox} expression, only the slowest mobility complex C4, was observed (Fig. 3A, lane 1).

Strikingly, induction of differentiation in the myeloid cells resulted in a significant shift in the pattern of complex formation (Fig. 3A). DMSO-induced differentiation of PLB985 cells resulted in greatly enhanced formation of complex C1, as well as formation of complexes C2, C3, and C4 (Fig. 3A, lane 3). DMSO-differentiated HL60 cells demonstrated enhanced formation of C4, with significant decrease in C1 formation, and slight decreases of C2 and C3 formation (Fig. 3A, lane 5). Quantitation of these changes is summarized in Table 1. PMA-differentiated U937 cells showed increased formation of complexes C2 and C3, with decreased formation of C1 (Fig. 3A, lane 7).

The EMSA and competition experiments described here demonstrate that a protein(s) with a similar binding specificity to PU.1 binds in multiple forms at the p47^{phox} promoter PU.1 consensus site in myeloid and B-cell lines. In order to determine whether PU.1 was a

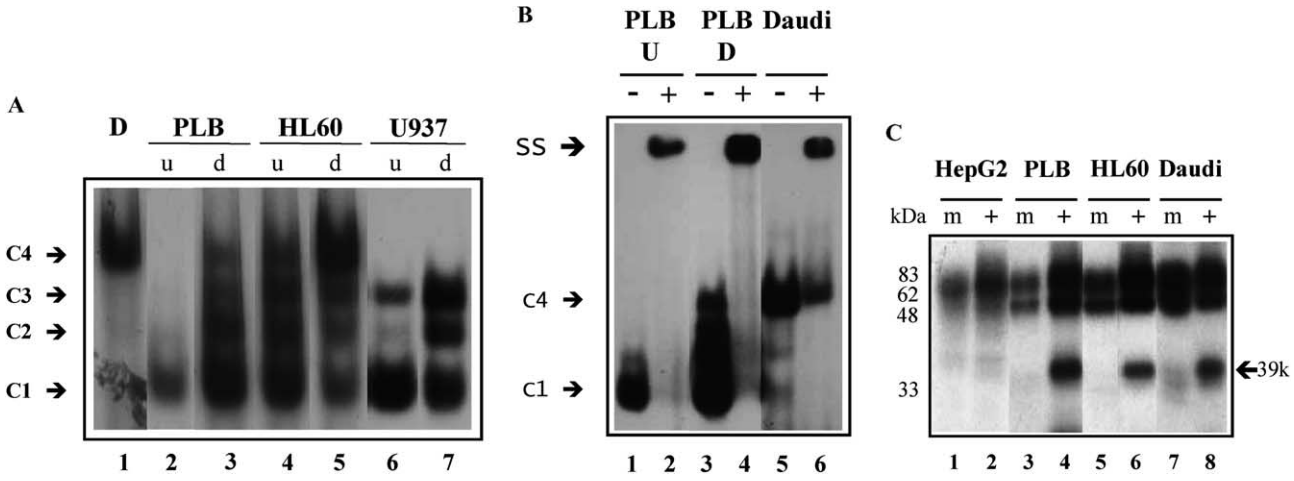

Fig. 3. EMSA complexes alter in response to myeloid differentiation and all contain PU.1. (A) Myeloid cells were differentiated for 48 hours to a granulocytic phenotype in the presence of DMSO (PLB985, HL60) or to a macrophage-like phenotype in the presence of PMA (U937). EMSA was performed with $p47^{phox}$ oligonucleotide probe P and 3 μg nuclear extract from Daudi B-lymphoblastoid cells (lane 1), undifferentiated PLB, HL60, and U937 (u, lanes 2, 4, and 6) and DMSO-differentiated PLB985 cells or HL60 cells (d, lanes 3 and 5), and PMA-differentiated U937 cells (d, lane 7). Specific complexes formed are indicated as C1, C2, C3, and C4. (B) EMSA was performed with $p47^{phox}$ oligonucleotide probe P (lanes 1–4) and 3 μg nuclear extract from undifferentiated PLB985 (PLB U, lanes 1 and 2), DMSO-differentiated (48 h) PLB985 cells (PLB D, lanes 3 and 4), or Daudi cells (Daudi, lanes 5 and 6). The binding reaction was incubated in the presence (+, lanes 2, 4, and 6) or absence (−, lanes 1, 3, and 5) of 2 μg polyclonal anti-PU.1antibody for 1 h at 4 °C. Specific complexes C1 and C4 are arrowed; anti-PU.1 antibody supershift is indicated by SS. (C) Multiple EMSA bands are not multi-protein complexes. EMSA complexes were formed, as described in (A) using oligonucleotide P (+) or its non-binding mutant equivalent (m) and exposed to short wave ultraviolet light. The cross-linked proteins were separated on 12.5% SDS–polyacrylamide gels. The molecular weight of the complex is revealed by the migration of the radioactive DNA probe to which the proteins were bound. Nuclear extracts were as follows: HepG2, G2 hepatoma cells; PLB, undifferentiated PLB 985 cells; HL60, DMSO-treated HL60 cells; and Daudi, Daudi B cells. The arrow identifies the specific UV cross-linked complex.

Table 1
Representation of different PU.1 EMSA complexes in myeloid cell lines before and after differentiation

Band	Undifferentiated PLB 985	Differentiated PLB 985	Undifferentiated HL60	Differentiated HL60
C4	nd[a]	14.0 ± 0.2[b]	18.3 ± 0.5	45.1 ± 1.0
C3	nd	14.1 ± 1.1	20.0 ± 0.3	16.0 ± 1.4
C2	nd	27.2 ± 0.7	25.9 ± 0.2	16.7 ± 0.9
C1	100	44.8 ± 1.5	35.9 ± 0.6	22.2 ± 1.0

[a] Not detected.
[b] Values are mean percentage ± SEM ($n = 3$).

component of complexes C1–4, antibody supershift experiments were performed (Fig. 3B). All complexes formed on the oligonucleotide P with nuclear extract from undifferentiated (Fig. 3B, lanes 1 and 2) and differentiated PLB985 (Fig. 3B, lanes 3 and 4) myeloid cells and Daudi B-cells (Fig. 3B, lanes 5 and 6) were supershifted in the presence of polyclonal anti-PU.1 antibody (Fig. 3B, lanes 2, 4, and 6, respectively), but not by a control antibody (data not shown). These results demonstrate that EMSA complexes C1–4 all contain PU.1.

To resolve whether these multiple EMSA species were due to assembly of multi-protein complexes, we subjected the EMSA complexes to cross-linking with ultraviolet (UV) light. The cross-linked proteins were then separated on SDS–PAGE to assess the molecular weight of the complex. The proteins specifically binding oligonucleotide P were detected by the presence of the radiolabelled DNA in the complex. The specificity of the UV cross-linked complexes was tested by the use of the mutant oligonucleotide, Pmt (Fig. 3C, lanes 1, 3, 5 and 7) and a nuclear extract prepared from HepG2 hepatoma cells, that lack PU.1 (HepG2, Fig. 3C, lanes 1 and 2). A specific UV cross-linked complex of approximately 39 kDa was observed (Fig. 3C, lanes 4, 6, and 8), using either myeloid or lymphoid cell extracts. Moreover, the molecular weight of this complex did not alter regardless of whether the extract predominantly formed the EMSA complex, C1 (PLB, Fig. 3C, lane 4), or the slowest migrating EMSA complex, C4 (HL60 and Daudi, Fig. 3C, lanes 6 and 8, respectively). This was a clear indication that the PU.1 EMSA complexes C1 to C4 did not differ in protein composition.

EMSA complexes C1–4 are differentially phosphorylated

Previous investigations of PU.1 have suggested that its activity can be modulated by phosphorylation [17–19]. We first investigated whether the PU.1 EMSA complexes could be supershifted or disrupted using an anti-phosphoserine monoclonal antibody. Anti-phosphoserine monoclonal antibody 4A3 (from Affiniti Research, see Materials and methods) was found to disrupt the EMSA complexes causing a major reduction in complex formation (Fig. 4A, lane 2). In contrast, no disruption was caused by an antibody to the transcription factor IRF-1 (Fig. 4A, lane 3). The pattern of complex disruption with the anti-phosphoserine antibody, also differed from the PU.1 supershift (Fig. 4A, lane 4). With anti-PU.1 the slower migrating complexes were less efficiently supershifted, whereas the anti-phosphoserine antibody, preferentially disrupted the slower migrating (and presumably, more highly phosphorylated) complexes, particularly C4.

We extended this analysis by isolating the proteins specifically binding the P oligonucleotide. Differentiated HL60 cell nuclear extract proteins were incubated with biotinylated P oligonucleotide and the DNA–protein complex was captured with streptavidin-conjugated magnetic beads (μMacs). Proteins eluted from the beads were then Western blotted and reacted with either the anti-PU.1 antibody or the anti-phosphoserine antibody 4A3. The anti-PU.1 antibody identified a major band of approximately 39 kDa (Fig. 4B, lane 1). This band was preferentially retained by the P oligonucleotide (Fig. 4B, lane 3) but significantly depleted in the unbound material (Fig. 4B, lane 2). The same 39 kDa protein was also detected by the anti-phosphoserine antibody (Fig. 4B, lane 5), providing further evidence that the PU.1 in the extract was serine phosphorylated.

To add to these observations, we tested whether the multiple PU.1 containing EMSA complexes were products of differential phosphorylation by treating induced HL60 cell nuclear extract with calf intestinal alkaline phosphatase (CIAP) or potato acid phosphatase (PAP). Incubation with CIAP virtually eradicated the formation of all EMSA complexes on the P oligonucleotide (Fig. 5A). As we considered this a stringent treatment, we also incubated the complexes under milder conditions by titrating different amounts of PAP and demonstrated conversion of the slower migrating complexes to faster migrating ones (Fig. 5B).

As the −58 bp minimal promoter binds PU.1 solely, it seems reasonable to assume that the differentiation-induced increases in PU.1 phosphorylation are directly linked with the differentiation-dependent up-regulation of reporter gene transcription we observed.

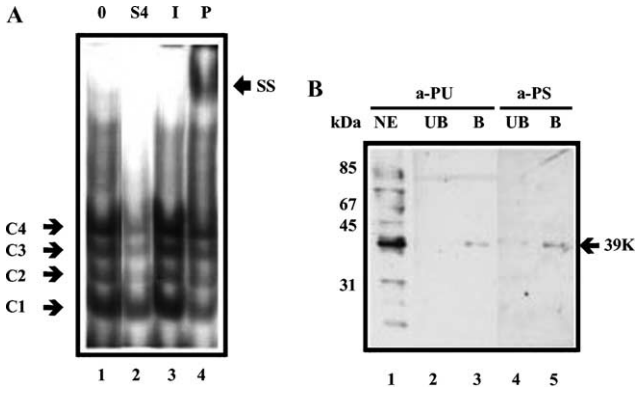

Fig. 4. Anti-phosphoserine antibody disrupts PU.1 EMSA complexes and reacts with PU.1 consensus-binding protein on Western blots. (A) EMSA complexes formed on P oligonucleotide with nuclear extract from differentiated HL60 cells pre-incubated without (0, lane 1) anti-phosphoserine monoclonal antibody 4A3 (S4, lane 2), antibody to IRF1 (I, lane 3), or PU.1 (P, lane 4). C1–C4 identify the differentially phosphorylated EMSA complexes; SS, anti-PU.1 supershift. (B) Western blot analysis of proteins captured on oligonucleotide P. DNA–protein complexes formed, as for EMSA analysis, on biotinylated oligonucleotide P were captured using streptavidin-conjugated magnetic microbeads and the bound proteins were eluted, separated on 12.5% SDS–polyacrylamide gels, and transferred to nitrocellulose membrane. The membranes were reacted with anti-PU.1 (a-PU, lanes 1–3), or anti-phosphoserine (a-PS, lanes 4 and 5) antibody and binding detected by chemiluminescent detection. NE, Total differentiated HL60 cell nuclear extract (2 μg); B, bound protein fraction, UB, unbound protein fraction. Black arrow identifies the 39 kDa PU.1 protein.

More highly phosphorylated forms of PU.1 display enhanced binding affinity

We further established that increased levels of PU.1 phosphorylation can be correlated with an in-

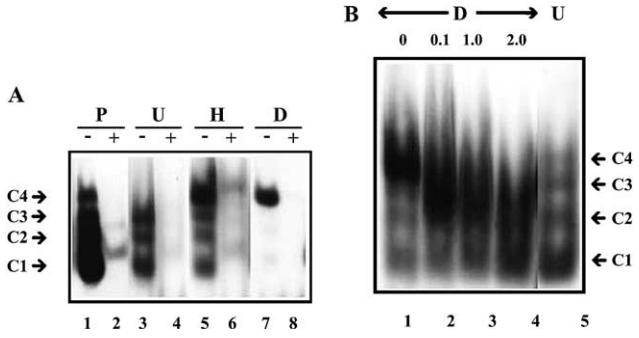

Fig. 5. Multiple PU.1 EMSA complexes result from differential phosphorylation. (A) EMSA complexes formed on the P oligonucleotide with nuclear extract from Daudi (D, lanes 7 and 8), DMSO-differentiated PLB985 (P, lanes 1 and 2), HL60 (H, lanes 3 and 4) or PMA differentiated U937 cells (U, lanes 5 and 6) were incubated at 37 °C with (+) or without (−) 1 μl of calf intestinal alkaline phosphatase (CIAP). (B) EMSA complexes formed on the P oligonucleotide with nuclear extract from DMSO-differentiated HL60 cells (D), were incubated with varying amounts (0–2 units) of potato acid phosphatase, as indicated. The zero phosphatase sample was incubated as for the other samples, but in the presence of heat-killed enzyme. Undifferentiated (U) HL60 cells are shown for comparison. The figure shows a typical example of this experiment, which was performed seven times.

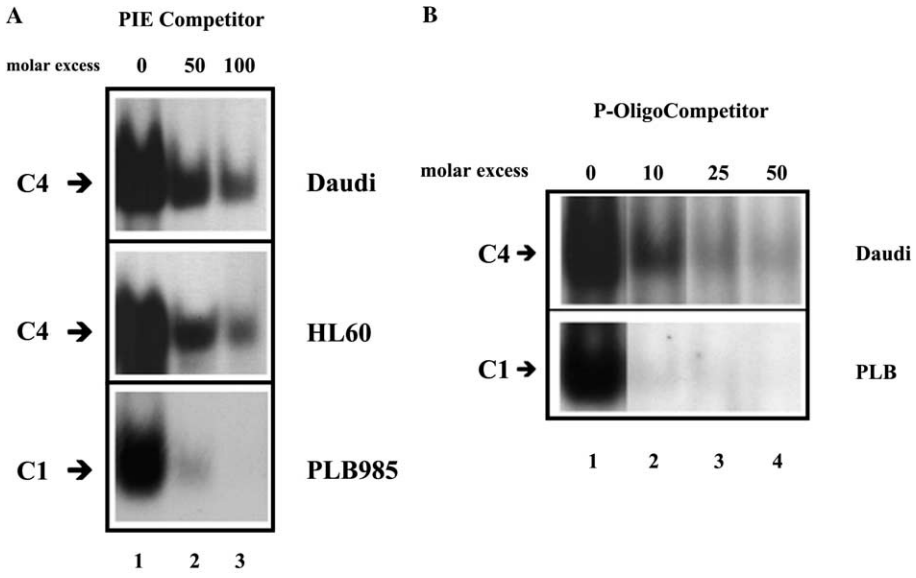

Fig. 6. Hyperphosphorylated forms of PU.1 have increased DNA binding affinity. (A) Competition of EMSA complexes C4 or C1 formed on the P oligonucleotide with Daudi (C4), HL60 (C4) or PLB985 (C1) cell nuclear extracts with excess unlabelled PIE oligonucleotide competitor, as indicated. (B) EMSA complexes C4 and C1 formed on the P oligonucleotide with Daudi and PLB985 cell nuclear extracts, respectively, were subjected to competition with an increasing molar excess of unlabelled cognate oligonucleotide, as indicated.

creased affinity for its consensus binding site. EMSA was performed using the P oligonucleotide as probe and the, lower affinity, PIE site as a competitor to compare the relative efficiency with which C4 and C1 were competed. C4 was found to be much more stable to competition than C1; a 50-fold excess almost completely removed the C1 complex (Fig. 6A, bottom panel), whereas at this excess, C4 was reduced to a much smaller degree (Fig. 6A, top and middle panels).

To extend this observation, complexes formed with nuclear extracts from Daudi cells, which contain almost exclusively complex C4 (the most highly phosphorylated form of PU.1), and undifferentiated PLB985 cells, which produce predominantly complex C1 (the least phosphorylated form), were subjected to competition with increasing excesses of unlabelled cognate P oligonucleotide. This clearly demonstrated that the more highly phosphorylated complex was more stable to competition. At a 10-fold excess the C1 complex was completely eradicated (Fig. 6B, lower panel), whereas the C4 complex remained significantly less affected with complexes still detectable at 50× excess of competitor (Fig. 6B, upper panel), indicating that the C4 complex was more tightly bound.

We conclude, therefore, that the up-regulation of $p47^{phox}$ transcription concomitant with terminal myeloid differentiation is mediated, in part, through a greater binding affinity of PU.1, which is itself dependent on differentiation-induced increases in the levels of PU.1 phosphorylation.

Discussion

Expression of the NADPH oxidase complex is predominantly restricted to terminally differentiated phagocytic blood cells. This is reflected by the expression pattern of the genes encoding oxidase components $p47^{phox}$ and $gp91^{phox}$, which are transcriptionally activated following induction of myeloid differentiation. We have investigated a minimal promoter of 58 bp of proximal $p47^{phox}$ sequence by reporter gene in stable transfectants and in vitro binding assays. Stable integration of reporter gene constructs into the host cell genome is likely to provide a more accurate reflection of endogenous regulatory mechanisms than can be provided by transient transfection and this has been borne out to some extent by our observation that in stably transfected lines this minimal promoter is capable of up-regulating reporter gene activity, following myeloid differentiation. Moreover, this increased transcriptional activity is associated with changes in PU.1 complex formation, which we have shown to be caused by increased levels of PU.1 phosphorylation. These more highly phosphorylated forms of PU.1 appear to have an increased affinity for the consensus binding site in the $p47^{phox}$ promoter.

The data presented here reveal that in myeloid and B-cells, PU.1 binds the $p47^{phox}$ promoter consensus binding site as four distinct species (C1–4), in a highly consistent cell type-specific pattern that altered significantly in response to myeloid differentiation, with a shift, in all cases, to less rapidly migrating forms. This

could have implied that multi-protein complexes were generated by interaction of PU.1 with other proteins, as has been observed elsewhere [20], or that modifications to PU.1 itself were occurring. The data presented here clearly favor the latter model, in which PU.1 is differentially phosphorylated and more highly phosphorylated forms are generated in response to cellular differentiation.

A previous analysis of differentiation in U937 cells has been associated both with changes in the pattern of PU.1 binding to DNA and in its phosphorylation [17]. In that report, however, the presence of the most highly phosphorylated form of PU.1 was correlated with terminal differentiation and loss of the transformed phenotype. Conversely, other authors have suggested that hyperphosphorylation of PU.1 is a reflection of chronic activation, indicative of the transformed state [21], or related to myeloid versus lymphoid tissue specificity [19]. None of these models is consistent with the observations described here. First, we have observed that the most highly phosphorylated form of PU.1 is present almost exclusively in the Daudi B-cell line, so it is clearly compatible with cell division and a transformed phenotype. Second, however, in myeloid cell lines hyperphosphorylation of PU.1 is associated with terminal differentiation and loss of the transformed phenotype. It would seem rather that PU.1 hyperphosphorylation is associated with the high level transcription of $p47^{phox}$ seen in differentiated myeloid cells and in the Daudi B-cell line.

The striking change in PU.1 complex formation, we observed, following induction of myeloid differentiation is presumably also of functional relevance, since it correlates closely with up-regulation of reporter gene activity in stably transfected myeloid cells. Similar changes in the PU.1 DNA binding pattern have also been observed in c-fes activation of PU.1 in U937 cells [22]. This view would also be consistent with the observation that LPS-induced phosphorylation of PU.1 at serine 148 increases the transactivation function of PU.1 in macrophages [18]. Evidence for phosphorylation-mediated enhancement of PU.1 binding through increased affinity provides an attractive model for the correlation of complex changes induced by myeloid differentiation with up-regulated promoter function and would be consistent with the observation of increased PU.1 binding in macrophages in response to LPS induced hyperphosphorylation [23], but would run somewhat counter to the view that PU.1 phosphorylation is not required for DNA binding activity but affects only its transactivation function, as has been suggested elsewhere [19]. We have found however, that high levels of phosphatase treatment do seem to eradicate PU.1 related EMSA complexes, which is also consistent with our data on the increased binding affinity of PU.1 in its hyperphosphorylated state.

Notwithstanding the changes in PU.1 binding affinity, we have observed on myeloid differentiation, competition analysis at the $p47^{phox}$ PU.1 consensus binding site has shown that it is already a high affinity binding site, significantly greater than the PIE site from the FcRγIb gene, or HAF from $gp91^{phox}$. Indeed, it is possible to derive a hierarchy of affinities highly consistent with the flanking sequence requirements previously described for PU.1 binding [24].

It is obviously of importance to establish the sites within PU.1 that are the targets for serine phosphorylation. Interestingly, two PU.1 proteolytic cleavage products, we observed in some preparations of nuclear extract, were reactive with the anti-PU.1 antibody, raised against a C-terminal peptide, but were not reactive with the anti-phosphoserine antibody, suggesting that the sites of phosphorylation were localized to the N-terminal region of the protein. This would probably rule out ser143, a casein kinase target in the PU.1 "PEST" domain, but would focus attention on the three serine residues located in the acidic transactivation domain (amino acids 33–74). Of these, ser41 has been implicated in the increased transcription mediated by the κE3′ enhancer, in B cells activated by Ras-phosphoinositide 3-kinase-dependent externally regulated kinase, AKT [25].

The identity of the kinase responsible for the differentiation-dependent phosphorylation of PU.1 is also of interest. Casein kinase II has been demonstrated to phosphorylate PU.1 at appropriate sites [18,26], but a role for protein kinase C as described previously in macrophages [23] seems not unreasonable in the context of myeloid cell differentiation.

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