

Genomic Structure, Chromosomal Localization, Start of Transcription, and Tissue Expression of the Human p40-phox, A New Component of the Nicotinamide Adenine Dinucleotide Phosphate-Oxidase Complex

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p40-phox is a newly isolated cytosolic component of the nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase that copurifies with p67-phox. Although its function is not well defined, preliminary evidence indicates that it is a component of the cytosolic complex. We report the characterization of the human p40-phox gene, which is single copy and spans approximately 18 kb with 10 exons. Based on fluorescent in situ hybridization (FISH) studies and analysis of somatic hybrid cell lines, the chromosomal location of p40-phox is human chromosome 22q13.1. The start of transcription has been mapped to bp -156. The expression of

p40-phox message is restricted to hematopoietic cells. In addition to identifying the mRNA transcript on Northern blot analysis in cells known to express components of the NADPH-oxidase, polymorphonuclear leukocytes, monocytes, B lymphoblastoid cell lines, and eosinophils, p40-phox is also expressed in two other cell types of white cell lineage, mast cells, and basophils. In addition, the mRNA for p40-phox is expressed in megakaryocytic cells, but not in erythroid cells.

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THE NICOTINAMIDE adenine dinucleotide phosphate (NADPH)-oxidase is a multicomponent enzyme system responsible for the oxidative burst in which electrons are transported from NADPH to molecular oxygen, generating reactive oxidant intermediates.^{1,2} The NADPH-oxidase is dormant in resting cells, but upon activation is assembled at the membrane.³ At the time of activation, the cytosolic complex associates with the membrane subunits of the cytochrome b-245, gp91-phox, and p22-phox.³ Studies of patients with chronic granulomatous disease, (CGD), an inherited disorder of phagocytic cells, have unequivocally established the importance of at least two cytosolic factors, p47-phox and p67-phox.⁴⁻¹⁰ The absence of either prevents generation of superoxide by the NADPH-oxidase and clinically results in the CGD phenotype.¹¹⁻¹³ Several other cytosolic components, which may participate in the activity of the NADPH-oxidase, have been identified and include a small G protein (known as rac2 in humans), rho-GDI, and p40-phox.¹⁴⁻¹⁹ In the cell-free system, which is used to measure the end stage signal transduction and assembly of NADPH-oxidase factors at high concentrations, p40-phox and rho-GDI are not absolutely required, whereas p47-phox, p67-phox, and either of the rac proteins, rac 1 or rac 2 are needed.^{20,21}

p40-phox, a new component of the NADPH-oxidase, was initially isolated from the cytosol of neutrophils and monocytes.^{19,22} The published cDNA sequence of p40-phox shows a 1.2-kb message that encodes for a protein of 339 amino acids.¹⁹ Although the specific function of p40-phox has not been defined, several observations suggest that it may be important for the assembly and/or activation of the NADPH-oxidase complex in whole cells. During partial purification of the cytosolic oxidase components with gel-filtration and anion-exchange chromatography, p40-phox was eluted with a 250 kD complex. The three proteins, p47-phox, p67-phox, and p40-phox remained complexed during the anion-exchange chromatography. The p67-phox/p40-phox complex can be immunoprecipitated from normal and p47-phox-deficient cytosol. In addition, the relative amount of p40-phox protein detected in p67-phox-deficient CGD patients was substantially reduced, leading the investigators to suggest that p40-phox primarily associates with p67-phox.¹⁹

Analysis of the nucleotide and amino acid sequence of p40-phox indicates homology with the N-terminus of p47-phox.¹⁹ More importantly, p40-phox has an SH3-like domain that shares approximately 30% homology with the SH3 domains of both p47-phox and p67-phox. SH3 domains have been reported to be putative binding sites for p47-phox and p67-phox in the assembly of the NADPH-oxidase.²³⁻²⁵ The SH3 domain of p40-phox binds to p47-phox in the C terminal region, but not its SH3 domain and interacts with the p67-phox in the yeast two hybrid system, providing evidence that p40-phox may be important for the assembly of the NADPH-oxidase system.²⁶

The gene structure for four genes of the NADPH-oxidase, gp91-phox, p22-phox, p47-phox, and p67-phox has been published.²⁷⁻³⁰ Recently, it has been determined that the p47-phox gene has 11 exons and not 9, as originally reported.³¹ The identification of the gene structures has permitted detailed molecular analyses of CGD patients of the four classical types. To date, analysis of CGD patients throughout the world has shown that three of the four types of CGD display a heterogeneity of mutations scattered throughout the genes of gp91-phox, p67-phox, and p22-phox.¹² The molecular epidemiology of p47-phox-deficient CGD differs from the other three in that nearly all patients studied are homozygous for a GT deletion at the beginning of exon 2.³⁰⁻³⁵

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Here we report the structure of the gene for p40-phox and demonstrate its location on human chromosome 22q13.1. The immediate 5' upstream region has been characterized and the start of transcription has been mapped to bp -156 (relative to the start of translation). The tissue specificity of the p40-phox gene expression is restricted to hematopoietic cells. We have determined that p40-phox is expressed at the message level in mast cells, basophils, eosinophils, and also in megakaryocytes. Furthermore, p40-phox mRNA is detectable in early undifferentiated myeloid cell lines.

MATERIALS AND METHODS

Isolation of genomic clones for the p40-phox gene. Genomic clones of the human p40-phox gene were isolated by screening a human genomic library (Embl3A) with a full-length cDNA probe labeled by random primer method. First strand cDNA was synthesized from RNA extracted from normal human monocytes using a commercial kit (Boehringer Mannheim, Indianapolis, IN) with avian myeloblastosis leukemia virus reverse transcriptase (RT) (C. Carter, Department of Transfusion Medicine, National Institutes of Health, Bethesda, MD). The cDNA probe for the screening of the genomic library was generated by polymerase chain reaction (PCR) using two unique oligonucleotides ATGGCTGTGGCCAGCAGCTG and TCATGGCATCGTGTGTAGACCCT from first-strand cDNA. The buffers and concentrations were used according to the manufacturer (Perkin-Elmer, Norwalk, CT). The conditions for the PCR reaction were as follows: 30 cycles of 94° for 1 minute, 55° for 1 minute, 72° for 2 minutes, followed by a 7-minute extension at 72°C. The PCR product was subcloned into the TA-vector, pCRII (Invitrogen, La Jolla, CA). The sequence was confirmed by the dideoxynucleotide chain termination method and compared with two separate clones isolated during the initial screening reported by Wientjes.¹⁹ A total of 1.5×10^6 clones were lifted on nitrocellulose filters and hybridized overnight at 42°C in 50% formamide, 6 × SSC (saline sodium citrate solution), 50 mmol/L NaPO₄ pH 7.0, 0.1% sodium pyrophosphate, 6 × Denhardt's solution, 0.1 mg/mL salmon sperm DNA, and 0.1% sodium dodecyl sulfate (SDS). Four consecutive washes were performed at 42°C in 2 × SSC, 1 × SSC, 0.5 × SSC, and 0.2 × SSC. Two separate genomic phage clones were purified and analyzed by Southern blot analysis using unique, end-labeled oligonucleotide probes corresponding to the published cDNA sequence and by direct sequencing of subcloned fragments.

A single P1 clone was isolated from a diploid human P1 library (Human Genome System, Inc, St Louis, MO) by a PCR-based method of screening with two oligonucleotides, F7, GTGAAGATCCTCAAAGACTT and R6, GAGATCTTCCTCCACCGCA, which generated a 380-bp fragment spanning exon 8/intron 9. Sequencing of this clone was performed to confirm intron/exon boundaries using two different methods, direct sequence analysis and sequence analysis of subcloned fragments of P40-1 into either pBluescript KS+ (Stratagene, La Jolla, CA) or pCR-II (Invitrogen). A preliminary restriction map was constructed and used to confirm the findings of the analysis of the phage clones.

Analysis of genomic map and intron-exon borders. Intron-exon borders were identified by DNA sequence analysis using dideoxynucleotide chain termination method. Intronic sequences were confirmed by both Southern blot analysis and PCR-generated fragments, which were subcloned and sequenced using oligonucleotides generated during the analysis.

Mapping the start of transcription. Primer extension was performed as follows. An oligonucleotide, PR4, TCACCTCTCACTTCCTCCAGCCAC complementary to the sense cDNA strand be-

ginning at bp -61, upstream of the start site of translation, was labeled with ³²P-adenosine triphosphate (P-ATP) and polynucleotide kinase to a specific activity of approximately 1×10^9 cpm/μg. A total of 5×10^5 cpm of probe was hybridized to total RNA from normal human monocytes and dimethylformamide (DMF) induced PLB-985 cells at 50°C for 1 hour in 12 μL of 100 mmol/L KCl/10 mmol/L MgCl₂/25 mmol/L Tris HCl pH 8.5.³⁶ The reverse transcription reaction was initiated in a buffer containing 30 mmol/L KCl, 8 mmol/L MgCl₂, 50 mmol/L Tris HCl (pH 8.5), 500 μmol/L dNTP, 25 mg of actinomycin D per mL, 4 U of RNasin and 50 U of avian myeloblastosis virus reverse transcriptase for 90 minutes at 42°C in a shaking bath. After ethanol precipitation, samples were resuspended in a formamide buffer, boiled, and analyzed on a 6% acrylamide/8 mol/L urea gel.

Northern blot analysis. Total RNA was isolated using RNAzol (Tel-Test, Inc, Friendswood, TX) from 18 different human tissues (Clontech, Palo Alto, CA). Further characterization of the expression of hematopoietic cells was performed with total RNA extracted from normal human monocytes, neutrophils, and eosinophils. RNA was extracted from the following cell lines, two separate B lymphoblastoid cell lines (one derived from a normal individual and a patient with p47-phox-deficient CGD), three myeloid cell lines, U937, THP-1, and PLB-985, the T-cell line H9, basophilic-derived cell line KU 802, mast cell-derived cell line, HMC1, the erythroleukemia cell line, K562 (treated and untreated with hemin), and DAMI (a human megakaryocytic cell line).³⁷⁻⁴⁶ In addition, we also analyzed RNA harvested from KU802 and HMC1 cells treated with phorbol myristate acetate (PMA) and the ionophore, A23187, for 4 hours. All RNA samples, except the eosinophils (which were studied by a RT-PCR assay), were evaluated by Northern blot analysis. A total of 20 μg per lane of total RNA was loaded onto a denaturing 1% agarose gel, electrophoresed, and transferred to N+ Hybond (Amersham, Arlington Heights, IL) and ultraviolet (UV) cross-linked. The RNA blots were hybridized overnight with random primed p40-phox cDNA probe and washed according to the following conditions, all at 42°C, in 2 × SSC, 1 × SSC, and 0.2 × SSC for 20 minutes each. Afterwards, autoradiography was performed.

Chromosomal localization. Aliquots of DNA from human-rodent hybrid cell lines containing different human chromosome(s) (Coriell Institute for Medical Research, Camden, NJ) were screened by PCR, using primers F7 and R6 under the conditions described earlier. The subchromosomal map position was assessed by fluorescent in situ hybridization (FISH) analysis. Metaphase spreads were prepared from methotrexate-synchronized and 5-bromodeoxyuridine (BrdU)-treated cultures⁴⁷ of a healthy donor. Colcemid treatment, hypotonic incubation, and fixation in methanol/acetic acid followed standard protocols.⁴⁸ P1-DNA was labeled with biotin-16-dUTP (Boehringer Mannheim) by nick-translation. A total of 100 ng of labeled DNA were precipitated in the presence of 5 μg of Cot1-DNA and 10 μg of salmon sperm DNA, resuspended in 10 μL hybridization solution (50% formamide, 2 × SSC, 10% dextran sulfate), denatured for 5 minutes at 76°C, and preannealed for 30 minutes at 37°C. The probe was then added to previously denatured metaphase chromosome preparations, hybridized, and detected using avidin-FITC (Vector Laboratories, Burlingame, CA). The Brd U-induced R-banding pattern was generated through a fluorescence photolysis step, followed by staining with propidium iodide.⁴⁹ Fluorescence signals were acquired using a cooled CCD-camera (Photometrics, Tucson, AZ), mounted on a Leica DMRBE-microscope, and visualized using Gene Join.

Subregional localization of p40-phox was performed with a PCR assay using 15 somatic cell hybrids from a panel of 26 hybrid cell lines. Primers, p40-phoxF, GGACATAGCTCTGAATTACCGG and p40-phoxR, GGCATCGTGTGTAGACCCT, were designed using

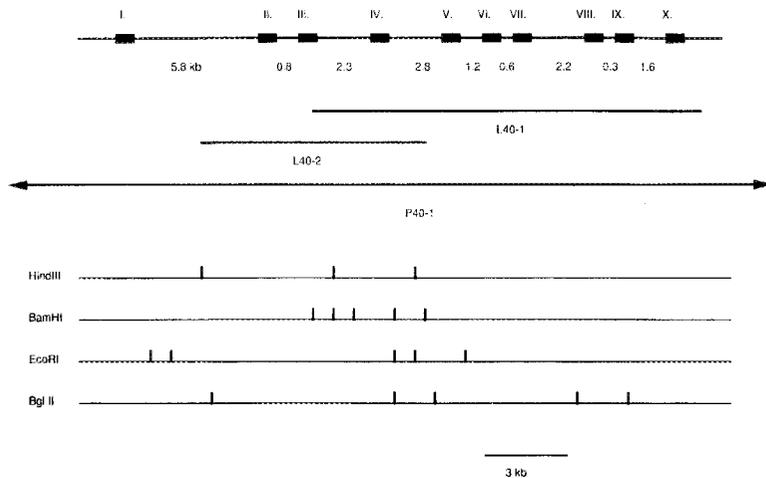


Fig 1. Gene structure for p40-phox with a partial restriction map. The bacteriophage clones were isolated from a human genomic library using a full-length p40-phox cDNA probe. The P1 clone was isolated by a PCR technique described in the text.

the program PRIMER (M.J. Daly, S. Lincoln, and E.S. Lander, Whitehead Institute, Cambridge, MA, 1992) and were used to amplify a 174-bp fragment surrounding exon 10. Conditions for the PCR reaction were as follows: 95°C for 5 minutes, 35 cycles of 94°C for 15 seconds, 62°C for 15 seconds, and 72°C for 1 minute 22 seconds, and a final 7-minute extension at 72°C. The PCR products were electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide staining.

RESULTS

Isolation of genomic clones and mapping of p40-phox gene. Two overlapping recombinant Emb13A bacteriophage clones, L40-1 and L40-2, were isolated from a human genomic library by screening with a full-length cDNA probe containing the coding region of p40-phox mRNA (Fig 1). A

single P1 clone, P40-1, was obtained and its analysis by Southern blot and sequencing of PCR products confirmed the map generated by the overlapping phage clones. P40-1 extends far upstream of the start of transcription. Analysis of these three clones resulted in a composite map of selected restriction sites, confirmed by genomic Southern blots (Fig 1). Exon positions were determined by sequencing of sub-clones either directly isolated from the phage clones or generated by PCR using unique oligonucleotide primers identified during the digests. Southern blot analysis of normal DNA did not indicate large rearrangements or deletions (data not shown).

One bacteriophage clone, L40-2, contains a 7-kb insert that extends from intron 1 to intron 4 and thus, does not

Table 1. p40-phox Intron-Exon Boundary Sequences

5' Boundary	Intron Length	3' Boundary
I. CGG GCC GAG AG 117	gtgagtgcgggggtgtggcggccc-5.8kb-	atctcttttcccctccttcgcacag T GAC TTT 118
II. ACC AGC CAC TTT 271	gtaagacagactctatcttaccac-0.8kb-	tcccacacaacctctgtcctccttag GTT TTC GTC 272
III. ACA CTC CCA G 342	gtaggcggcactcccgtcctgctg-2.3kb-	aggacagctctttgtctcttctcag CC AAA GTC 343
IV. GCC TAC ATG AAG 470	gtaccagtgggccttgcaccttgg-2.8kb-	cggttctgctgtctcaccacacag AGC CTG CTC 471
V. ACC CGG AAA GT 528	gtaagtgaccagccccctggcttc-1.2kb-	acaagccccctgtctctctccacag C AAG AGC 529
VI. CCG AGA GCA GAG 627	gtaacccccggccccggctggcca-0.6kb-	ctccttactgcacgcttctcctcag GCT CTA TTT 628
VII. GAC TGG CTG GAG 758	gtgagttcagaagtgaggatggag-2.2kb-	tggcatcccttctcttctctctcag GGC ACT GTC 759
VIII. AGC ACC ATC AA 824	gtctgtggcctggaggaggggc-0.3kb-	tcactccagcctgtcaccctcttag G GAC ATC GCG 825
IX. GAG CTC ACA AG	gtagggggctgggaatggggctg-1.6kb-	attatccctgacttttccatgcag G CGG GAG TTC

Exonic sequence is indicated in upper case letters and intronic sequence is indicated in lower case letters. Numbers above each exon refer to the cDNA sequence beginning with the start site of translation. The sizes of the introns are approximate and have been confirmed by both PCR analysis and Southern blot analysis.

include either the start site of transcription or translation. The 3' clone, L40-1, contains a 13-kb insert, overlapping L40-2 by 4 kb between intron 3 and 4 and extends approximately 3 kb into the 3' untranslated region.

Exon-intron structure of coding region of p40-phox. The structure of the coding region of the gene for p40-phox was determined by analysis of the phage clones and the P1 clone. Sizes of exons and introns were confirmed by PCR amplification of genomic DNA and fragments subcloned in the TA-vector, pCRII. The gene spans approximately 18 kb and is divided into 10 exons (Fig 1). The exon-intron boundaries were identified by direct sequencing of either bacteriophage clones or PCR amplified fragments derived from L40-1, L40-2, and P40-1 (Table 1). All splice junction sequences conform to the GT/AG rule. The complete sequencing of all 10 exons confirms a product of 339 amino acids, as predicted by the translation product of the published cDNA sequence. The sizes of polypeptide regions encoded by the individual exons ranged from 11 to 64 amino acids, with the last exon encoding for the largest span of amino acids. Of note, the SH3 region, which may play a critical role in interactions with other components of the NADPH-oxidase, is distributed between three exons, 6, 7, and 8. Introns varied in size from 300 bases (intron 8) to 5.8 kb (intron 1).

During our analysis, we determined an error in the published cDNA sequence for p40-phox in the 5' upstream region.¹⁹ The previously published sequence from -60 to -130 is incorrect with respect to the orientation of the 5' upstream sequence. Its position relative to flanking sequences could not be verified in our genomic clone or in PCR amplification of this region from normal human genomic DNA. A second cDNA clone from the original screening was obtained and analyzed. Its sequence matched the genomic P1 clone isolated, as well as material amplified from normal human genomic DNA. The sequence for the open-reading frame was identical to the previously published sequence. On further analysis, the published sequence from

-60 to -128 matches in retrograde complement the correct sequence from -28 to -96. We believe this represents a cloning artifact. Figure 2B shows the corrected sequence (underlined) seen in the second cDNA clone, the genomic clone, and material amplified from normal genomic DNA.

Chromosomal localization of p40-phox. Screening DNA from human-rodent hybrid cell lines containing different human chromosome(s) with a p40-phox-specific PCR assay indicated that the gene maps to human chromosome 22 (data not shown). The subchromosomal mapping position of the P1-clone, P40-1, was determined by FISH to high-resolution

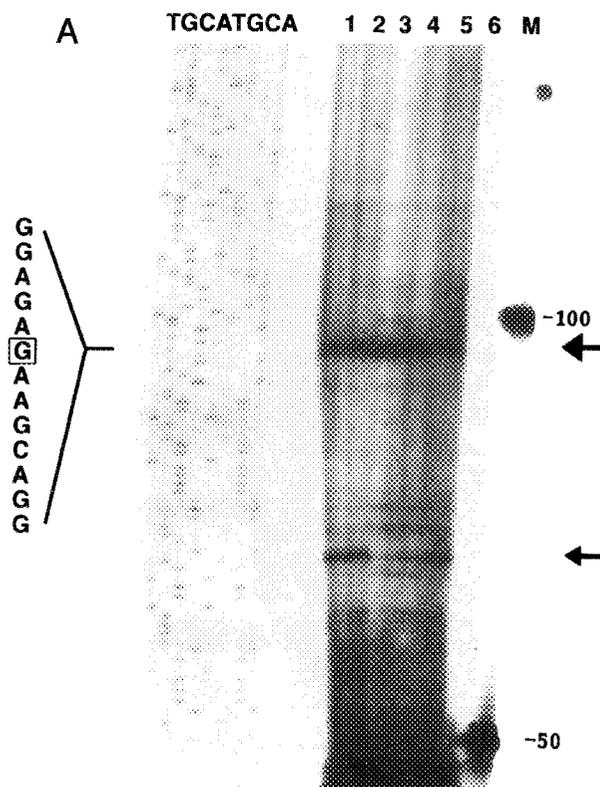
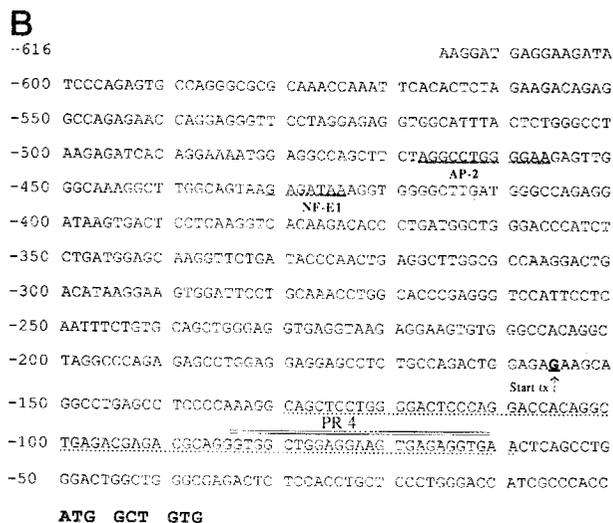


Fig 2. (A) Identification of the start of transcription by primer extension assay. Samples were run on 6% acrylamide/8 mol/L urea gel. The first eight lanes show a duplicate sequence analysis of the region above the start of translation using the primer PR4 in the following orientation T, G, C, A. Two blank lanes separate the sequence analysis from the primer extension assay, which is shown in lanes 1 to 6. Lane 1, total RNA purified from normal monocyte donor no. 1; lane 2, total RNA purified from normal monocyte donor no. 2; lane 3, total RNA from DMF-induced PLB-985 (preparation no. 1); lane 4, total RNA from DMF-induced PLB-985 (preparation no. 2); lane 5, total RNA from DMF-induced PLB-985 (preparation no. 3); lane 6, tRNA control, lane M denotes the RNA Century Marker (Ambion, Inc, Austin, TX). The major site for the start of transcription is indicated by the upper arrow. The lower arrow illustrates a possible secondary site. (B) 616 base pairs of the 5' upstream sequence for p40-phox. The start of transcription is indicated by an arrow. Consensus binding site for AP-2 and NF-E1 are underlined at bp -457 to -468 and -425 to -431 according to the start of translation. A correction of the published cDNA sequence¹⁹ for the 5' upstream region is highlighted with a dotted underline and includes the region corresponding to -60 to -130 from the start of translation. The primer, PR4, used for sequence analysis and primer extension analysis shown in (A) is indicated.



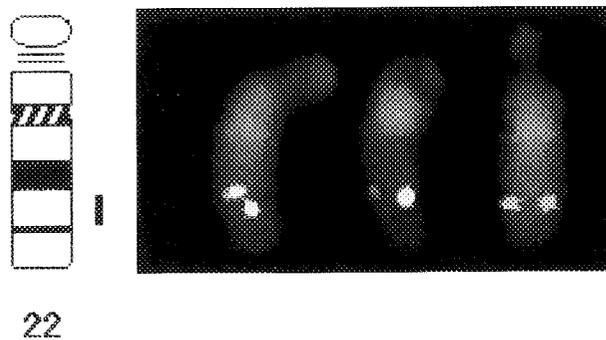


Fig 3. Hybridization of the biotinylated P1 clones for the p40-phox gene to a human metaphase spread. The DNA probe was detected with avidin-FITC and is pseudocolored in yellow. The chromosomes are counterstained with propidium iodide and pseudocolored blue. The BrdU-induced R-banding pattern allows for the chromosome and band identification. The probe hybridized to chromosomal map position 22q13. Ideogram of chromosome 22 displays schematically the G-banding pattern with the 550 band resolution. The bar besides the ideogram denotes the chromosomal mapping position of the P1-clone.

banded chromosomes. To generate a simultaneous fluorescent banding for gene mapping studies, we followed the methotrexate synchronization procedure with subsequent BrdU incorporation. This procedure shows an R-banding pattern and allows a band assignment up to the 550 band level. Fifteen metaphases were investigated: 12 displayed two hybridization signals on both homologues of chromosome 22q13, while three metaphases showed incomplete hybridization, (ie, only one hybridization signal on both homologues or two signals on only one homologue). Thus, the chromosomal map position of the P1 could be determined precisely when highly extended chromosomes were evaluated. For fine mapping, we used exclusively chromosome 22 homologues, which were at least 5 μm in length. The signal consistently mapped to chromosome band 22q13 (Fig 3).

Subregional localization of p40-phox on chromosome 22 was accomplished using a subset of 15 somatic cell hybrids from a panel of 26 hybrid cell lines. The extended panel divides chromosome 22 into 25 regions or bins and has been used to map over 300 markers to unique locations on the chromosome.⁵⁰ The p40-phox primer pair, p40-phoxF, and p40-phoxR (see Materials and Methods), gave a positive signal in the following cell lines: GM10888, GM11220, Cl-9/5878, AJO9, RAJ5BE, Cl 1-1/TW, Cl 15-1/PB and no specific signal for the following cell hybrids: GM11685, Cl-4/GB, Rad-37a, 514 AA2, GM11221, D6S5, Cl-3/5878, and Cl 21-2/PB (47A).⁵⁰⁻⁵⁶ These results map p40-phox below the hybrid breakpoint in D6S5 and above the one in Cl-3/5878, which corresponds to bin 15 of the extended somatic cell hybrid panel.⁵⁰ Using this PCR amplification technique, we have independently confirmed the FISH data and provide greater resolution of assignment to the region, 22q13.1, using the breakpoints of the somatic cell hybrids.

Characterization of the start of transcription. To define

the transcriptional initiation site, primer extension analysis was performed and identified a major start site. The start of transcription maps to purine G, located at base pair, -156, upstream of the ATG initiation of translation (Fig 2A). The band corresponding to bp 156 was seen consistently in six different experiments with RNA prepared from human normal monocytes, as well as RNA from differentiated PLB-985 cells. On two occasions, a second band was observed corresponding to the A at bp -129. All primer extension experiments were performed with the primer, PR4, internally labeled and complementary to the 5' end of the corrected p40-phox upstream sequence shown in Fig 2B (see below). A total of 616 base pairs sequence upstream of the start of translation are reported (Fig 2B). Sequence analysis of this region shows several notable features. There is no consensus sequence for either a TATA or CAAT motif. Three hundred and one bases upstream from the start of transcription is a consensus sequence for AP2 binding and 269 bases upstream of the start of transcription is an NF-E1 consensus binding site.

Tissue distribution of p40-phox. Northern blot analysis using a full length random primed p40-phox cDNA probe demonstrates that p40-phox mRNA is restricted to hematopoietic cells (Figs 4 and 5). p40-phox mRNA was detected in PMNs, monocytes, B lymphoblastoid cells, basophils, and mast cells by Northern blot analysis and eosinophils by RT-PCR (data not shown). In addition, the p40-phox mRNA is expressed in a megakaryocytic cell line, a cell type not previously demonstrated to express NADPH-oxidase components. p40-phox expression is associated with myeloid differentiation of human leukemia cell lines but interestingly, in the basophilic and mast cell lines, expression decreased following treatment with the combination of differentiation agents, PMA, and the ionophore, A23187. We analyzed the cell line PLB-985, which possesses biphenotypic potential (ie, differentiation into monocytes or neutrophils, with PMA or DMF, respectively). There was no significant increase in p40-phox mRNA levels following treatment for 4 days with either agent (Fig 5).

DISCUSSION

p40-phox is encoded by a single-copy gene that encompasses 18 kb and consists of 10 exons. Knowledge of the genomic structure will serve as the foundation for studying the regulation of p40-phox gene expression in hematopoietic cells. The localization by FISH of p40-phox to chromosome 22q has been established and the subregional localization has been accomplished using a PCR-based assay on a panel of somatic cell hybrids. Using PCR amplification of primers specific to exon 10, we have regionally mapped human p40-phox to 22q13.1. These results agree with and refine the FISH results. Unlike the p47-phox gene, the p40-phox gene appears to be a single copy.³¹ Other genes, which have been mapped to this region, include granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor β chain, histone H1 $^{\circ}$, hippocampal inward rectifier channel, interleukin-2 receptor beta, hemoglobin, and parvalbumin (Budarf et al, submitted).

Expression of p40-phox message is restricted to cells of

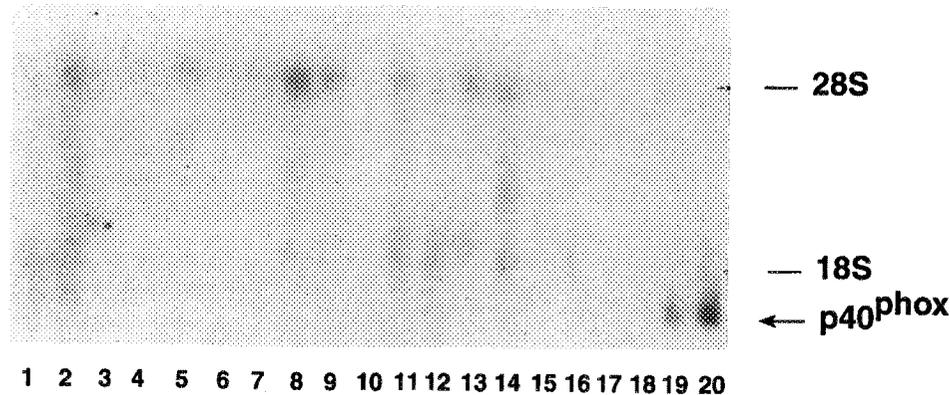


Fig 4. Northern blot analysis for tissue distribution of p40-phox. A total of 20 µg of total RNA (Clontech) was loaded per lane. The blot was hybridized with a full-length random primed p40-phox cDNA probe and washed as described in the text. Lanes 1, breast; 2, lung; 3, adrenal; 4, skeletal muscle; 5, kidney; 6, smooth muscle; 7, thymus; 8, testis; 9, prostate; 10, retina; 11, small intestines; 12, spleen; 13, ovaries; 14, pancreas; 15, heart; 16, brain; 17, uterus; 18, cerebellum; 19, B-lymphoblastoid cell line; 20, peripheral monocytes.

hematopoietic origin, neutrophils, monocytes, eosinophils, B lymphocytes, basophils, mast cells, and megakaryocytes. The pattern of expression of p40-phox included cells of the classical white cell lineage not known to generate large quantities of superoxide, mast cells, and basophils. In our study, we were unable to demonstrate the expression of the cytosolic NADPH-oxidase components, p47-phox, and p67-phox in basophils, mast cells, and megakaryocytes (data not shown). Expression of these two cytosolic factors was limited to neutrophils, monocytes, B lymphoblastoid cells, and eosinophils, all of which generate superoxide following activation of the NADPH-oxidase. p22-phox, (data not shown) was present in all cell types tested, as has been previously reported.^{27,57} Like p22-phox, p40-phox is abundantly expressed in an undifferentiated human leukemia cell line, PLB-985, and levels of mRNA do not significantly increase during differentiation. In this regard, we speculate that p40-

phox may participate in a function in addition to the NADPH-oxidase.

The major site for the start of transcription has been mapped to a single base pair, G at bp -156, relative to the ATG of the start of translation by primer extension. We cannot exclude the possibility of a second site at bp -129, shown in Fig 2A. However, we observed this band in only two of six experiments. Furthermore, the possible second site was not restricted to a specific source of RNA (either normal human monocytes or differentiated PLB-985 cells). The sequence surrounding the start site of transcription at -156 bp does not contain a consensus sequence for a TATA or CAAT box. Several GC rich regions are apparent. In this regard, the upstream region of p40-phox resembles p47-phox, which also lacks these DNA motifs in the immediate upstream region. Ongoing studies will hopefully characterize the *cis*-acting elements required for expression of p40-phox

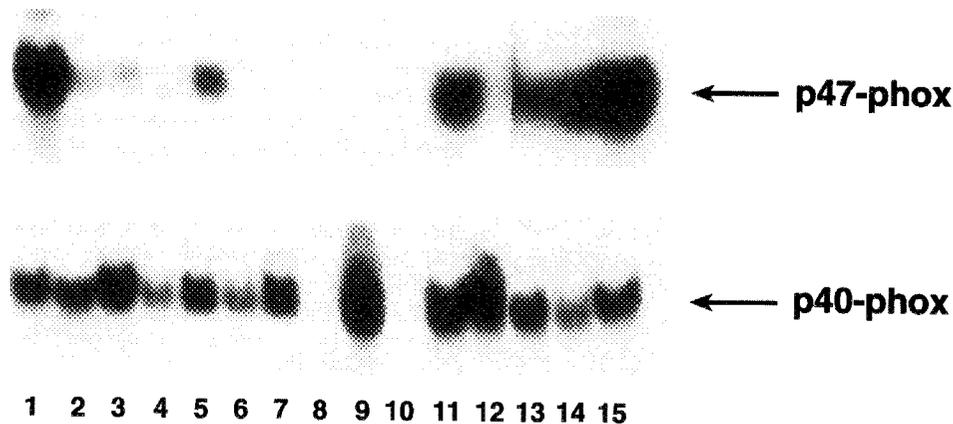


Fig 5. Expression of p40-phox in hematopoietic cells. A total of 20 µg of total RNA was loaded per lane and probed with a full-length random primed p40-phox cDNA as described in the text. Lanes 1, normal monocytes; 2, THP-1; 3, U-937; 4, HMC1 (PMA/A23187 treated) (mast cell); 5, HMC1 control; 6, KU802 (PMA/A23187 treated) (basophil); 7, KU802; 8, T-cell line; 9, DAMI; 10, K562; 11, PLB-985 (DMF induced); 12, PLB-985 control; 13, p47-phox-deficient B-lymphoblastoid cell line; 14, normal B-lymphoblastoid cell line; 15, normal monocytes. The top panel is probed with p47-phox cDNA and the bottom panel with p40-phox cDNA.

in hematopoietic cells, particularly in myeloid and monocytic cells.

Knowledge of the gene structure will be useful in determining if rare patients with a chronic granulomatous disease phenotype may have a mutation(s) in p40-phox gene. It will be particularly interesting to investigate patients with p67-phox deficient CGD who are reported to have decreased levels of p40-phox protein. Furthermore, the possible demonstration of a mutation in p40-phox gene would further establish the functional significance of p40-phox in the cytosolic complex of the NADPH-oxidase.

NOTE ADDED IN PROOF

p40-phox has been named neutrophil cytosolic factor 4 by the nomenclature committee of the Human Genome Database. The human symbol is NCF4.

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