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PU.1 Is Dominant and HAF-1 Supplementary for Activation of the gp91^{phox} Promoter in Human Monocytic PLB-985 Cells[1]

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Gp91^{phox} is a key component of the phagocyte NADPH oxidase. Mutations of its promoter found in patients with chronic granulomatous disease cause deficient binding of PU.1 and HAF-1. Because the two factors bind to the same site (Pu box) of the promoter, we attempted to clarify their relative *in vivo* contributions to activation of the gp91^{phox} promoter in monocytically differentiated PLB-985 cells using a dual luciferase reporter assay and a gel shift competition assay. We found that the activity of a series of single-point-mutated promoters increases or decreases according to an increase or decrease, respectively, in the affinity of the promoters to PU.1 but not to HAF-1. Two of 7 mutants showing weak binding affinity to PU.1 exhibited moderate promoter activity and normal binding affinity for HAF-1. These results suggest PU.1 is the dominant activator and HAF-1 is supplementary. The increased promoter activity of single-, double-, and triple-point-mutated constructs with sequences closer to that of the Ets-binding element correlates with their binding affinity to PU.1 but not to HAF-1, supporting that PU.1 is a more efficient activator than HAF-1. In contrast to co-expressed wild-type PU.1, dominant-negative PU.1 significantly inhibited the activity of a PU.1-optimised gp91^{phox} promoter construct. Therefore, we conclude that PU.1 and HAF-1 binding to the Pu box is dominant and supplementary, respectively, for activation of the gp91^{phox} promoter in human monocytic cells.

Key words: CGD, HAF-1, NADPH oxidase, PLB-985 cells, PU.1.

Gp91^{phox}, the counterpart of p22^{phox} in heterodimeric flavocytochrome b_{558}, is located in cytoplasmic and specific granule membranes as a component of the phagocyte NADPH oxidase (*1–3*). Upon phagocytic stimulation, gp91^{phox} in the integrated oxidase is assumed to transfer electrons from cytosolic NADPH to extracellular or phagosomal molecular oxygen to generate the superoxide anion, from which other antimicrobial reactive oxygen intermediates are formed (*4–8*). Because the gene (CYBB) of gp91^{phox} lies on the X chromosome (*9, 10–13* as reviews), a defect of it is the most common (approximately 70%) cause of chronic granulomatous disease (CGD) characterized by recurrent microbial infections due to deficient oxidase activity. As rare mechanisms causing X-linked CGD, we reported single base substitutions clustered around bp –55 of the gp91^{phox} promoter, like other authors (*14, 15*). These substitutions result in deficient expression of the gene in all or most neutrophils, monocytes, and B-lymphocytes, but not in eosinophils (*16*), suggesting that the former three kinds of cells have a common transcriptional regulatory mechanism that is impaired by the mutations (*14*).

The gp91^{phox} gene is exclusively expressed in terminally differentiated myeloid and B-lymphocytic lineage cells (*17–19*). The expression of the gene is further up-regulated, even in mature phagocytes, through mediators such as lipopolysaccharide, tumor necrosis factor α, granulocyte-macrophage colony-stimulating factor, and interferon γ (IFN-γ) (*20*). In contrast, the gene is reportedly down regulated by interferons α/β (*20*) and Human Granulocytic Ehrlichiosis agent (Rickettsia), an intracellular microbe (*21*). The latter mechanism may allow the microbe to grow freely in phagocytes. Therefore, the gene is situated at a key position for dynamic regulation of the phagocyte oxidase. Accordingly, analysis of transcriptional regulation of the gene will provide us with valuable information for a better understanding of host-defense mechanisms and for developing mole-

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Abbreviations bp, base pair(s), Pu box, PU.1/HAF-1 binding site, EMSA, electrophoretic mobility shift assay, CGD, chronic granulomatous disease, HAF-1, hematopoietic-associated factor 1, FBS, fetal bovine serum, TPA, tetradecanoyl phorbol 12-myristate 13-acetate; DTT, dithiothreitol, PCR, polymerase chain reaction, SD, standard deviation, IRF, interferon regulatory factor; ICSBP, interferon consensus sequence binding protein; PMSF, phenylmethylsulfonyl fluoride; DIC, dichloroisocoumarin, LUC, luciferase reporter gene, CMV, cytomegalovirus, PLB, passive lysis buffer; Wild, wild-type

cular devices to protect hosts from invasion by various pathogenic microbes.

Among the transcription factors regulating gp91^{phox} gene expression through *cis*-elements, we have focussed on PU.1 and HAF-1, because their binding is abolished by single base substitutions found in CGD (*14*), and these two share a common binding site (Pu box) at the gp91^{phox} proximal promoter. In contrast to the CCAAT displacement protein (CDP/cut), which may function at an immature stage of cells (*22*), these two factors may play a role in mature phagocytes, suggesting they are possible targets for modulation of the NADPH oxidase of macrophages in inflammation. PU.1 detected in the electrophoresis mobility shift assay (EMSA) is apparently a single protein and a member of the Ets family that requires 5′-NRCTTCCGGT-3′ as a consensus binding element (*23, 24*). HAF-1 is a complex containing Elf-1 (*25*), another Ets protein (*26*), and binds to the Pu box of the gp91^{phox} promoter (*14, 15, 25*) and an Ets-binding site of CD4 enhancer (*27*).

Although PU.1 and Elf-1-containing HAF-1 (*28*) were shown to bind to the Pu box of the proximal promoter of the human gp91^{phox} gene, their relative contributions to expression of the gene in human phagocytic cells has never been reported. The present results obtained by use of promoter constructs with systematically introduced point mutations and corresponding binding competitors strongly correlate the gp91^{phox} promoter activity much more with the binding of PU.1 than that of HAF-1

MATERIALS AND METHODS

Cell Culture—Myelomonoblastic cell line PLB-985 was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Asahi Technoglass, Funabashi) and 2 mM glutamine (Wako Life Science, Tokyo) at 37°C under a 5% CO_2 in air atmosphere.

Construction of Expression Plasmids with a Wild-Type gp91^{phox} Promoter and Its Point-Mutated Counterparts—The wild-type −105 to +12 bp gp91^{phox} promoter/firefly luciferase plasmid was constructed as described previously (*14*). In brief, a PCR product with sequences sensitive to *Kpn*I and *Bam*HI at its 5′ and 3′ ends, respectively, was digested with the two enzymes and then inserted into the *Bgl*II/*Bam*HI site of the pXP2N firefly luciferase gene vector. The nucleotide sequence of the insert was confirmed with a dideoxy terminating AutoRead Sequencing Kit II (Pharmacia/LKB, Uppsala, Sweden) and an A.L.F. DNA Sequencer II (Pharmacia/LKB). In order to prepare mutated promoter constructs, we first made pBluescript II KS (Stratagene, La Jolla, CA, USA) with the wild-type −105 to +12 bp gp91^{phox} promoter fragment as an insert located at its *Kpn*I/*Eco*RI site. Using this wild-type pKS plasmid as a template, we introduced a single-point mutation between bp −58 and −48 by overlapping PCR (*29*). Upstream and downstream duplexes were independently made using a set of 5′-outside forward 25-mer primers with a *Kpn*I-site and a reverse primer with a substituted base, and a set of forward primers with a complementarily mutated base and a 3′-outside reverse primer with a *Nar*I site, respectively. Secondary PCR was carried out using the mixture of the two above duplexes as templates and the above set of outside primers. The final PCR products were digested with *Kpn*I and *Nar*I, and then expanded as the insert of pKS in *Escherichia coli*. Plasmids with properly mutated sequences were digested with *Kpn*I and *Nar*I, and then ligated to the *Kpn*I/*Nar*I site of the pXP2N firefly luciferase gene vector. Dual-point- and triple-point-mutated plasmids were obtained in the same way, in that order, using single-point-mutated and dual-point-mutated plasmids, respectively. All PCRs were carried out with a Perkin Elmer GeneAmp® PCR System 9700 (Applied Biosystems, Norwalk, CT, USA) with a program (Ramp speed: 9,600); 95°C, 5 min as a starter, 35 cycles of 95°C for 30 s – 55°C for 30 s – 72°C for 1 min, and 72°C, 7 min for the final additional elongation.

Transient Expression Assays—Plasmids were purified with a Maxiprep kit (Qiagen Inc., Valencia, USA) and were transfected into PLB-985 cells as reported previously (*30*), but on a reduced scale. Briefly, 5×10^5 cells were suspended in 1 ml of 1640 RPMI medium containing 10% FBS and then treated with 1 μl of 160 μM TPA for 4 h at 37°C. Each 0.25 ml of the cell suspension of TPA-treated cells was transferred to the well of a 24-well multiwell-plate containing 1 μg of the gp91^{phox} promoter/firefly-luciferase-reporter plasmid, 15 nmol of TFL-01-Liposome (Daiichi Pharmaceutical, Tokyo), and 10 ng of cytomegalovirus (CMV) promoter/enhancer renilla luciferase plasmid (pRL-CMV) (Toyoink, Tokyo), as an internal control, and then kept at room temperature for 15 min and cultured for 16–22 h (*30*) at 37°C. TPA potentiated the efficiency of liposome transfection and induced monocytic differentiation of PLB-985 cells because the amount of gp91^{phox} mRNA was increased four times by TPA essentially as reported previously (*31*) The cells, harvested by centrifugation (12,000 rpm, 3 min), were suspended in 12.5 μl of 1× passive lysis buffer (PLB) (Promega, Madison, WI, USA) by vortexing (5 min), and then allowed to lyse completely for 15 min at room temperature on a rotary shaker. A 10 μl aliquot of the lysate clarified by centrifugation (12,000 rpm, 1 min) was used to assay the firefly and renilla luciferase activities with a PicaGene Dual Sea Pansy Luminescence kit (Toyoink) and a six-well luminometer, Berthold LB9505C (Berthold, Wildbad, Germany). In cotransfection experiments, 0 4 μg of murine wild-type or dominant-negative PU.1 expression plasmid kindly supplied by Dr. Klemsz (Indiana University, USA) (*32, 33*) was transfected with 1.6 μg of gp91^{phox} promoter-firefly-luciferase-reporter plasmid (−56C/−49T) and 25 ng of renilla luciferase plasmid into 2.5×10^5 PLB-985 cells suspended in 0 5 ml of the culture medium specified above.

Preparation of Nuclear Protein Extracts—Prior to nuclear protein extraction, PLB-985 cells were treated with 160 nM TPA for various times (typically 8 h) at 37°C. Nuclear extracts were prepared from the cells according to the method of Schreiber *et al* (*34*) with slight modifications. In brief, approximately 10^7 cells were allowed to swell in 400 μl of ice-cold buffer A [10 mM Hepes buffer (pH 7.9), containing 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma), 2 μg of leupeptin/ml (Sigma), 2 μg of aprotinin/ml (Sigma), 10 μg of pepstanin A/ml (Sigma), 10 μg of 3,4-dichloroisocoumarin (DIC)/ml (Boehringer Mannheim, Germany), and 10 μg of E-64/ml (Peptide Institute, Osaka)] for 15 min at 4°C. The cells were lysed with 25 μl of a 10% solution of Nonidet P-40 (Nacalai Tesque, Kyoto), and then vortexed vigorously for 15 s. The precipitate rich in nuclei

obtained from the lysate on centrifugation (12,000 ×*g*, at 4°C for 1 min) was suspended in 100 μl of ice-cold buffer C [20 mM Hepes (pH 7.9), containing 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 2 μg of leupeptin/ml, 2 μg of aprotinin/ml, 10 μg of pepstanin A/ml, 10 μg of 3,4-DIC/ml, and 10 μg of E-64/ml], and rotated for 30 min at 4°C. Nuclear extracts were separated by centrifugation (21,000 ×*g*, 4°C for 5 min) and the supernatant was frozen immediately in aliquots at –80°C Protein concentrations were spectrophotometrically determined with a Bio-Rad protein assay kit.

Electrophoretic Mobility Shift Assay (EMSA)—An EMSA probe was constructed from sense and antisense oligonucleotides identical to the fragment comprising position –65 to –41 of human gp91^{phox} in 400 μl of annealing buffer [100 mM Tris-HCl buffer (pH 8.0), containing 1.5 mM NaCl and 10 mM EDTA], denatured at 100°C, and then annealed at room temperature for 3 h. The double-stranded oligonucleotides were labeled with [α-^{32}P]dideoxyadenosine 5′-triphosphate (3,000 Ci/ml) with terminal deoxynucleotidyl transferase in a 3′-END labeling kit (Amersham International, Amersham, UK), and purified on ProbeQuant™ G-50 Micro Columns (Amersham Pharmacia Biotech, Tokyo). Five micrograms of nuclear protein extracts were incubated with a radiolabelled probe (10^4 cpm), equivalent to 3.0 fmol, on ice for 15 min in 20 μl of binding reaction mixture (Amersham Pharmacia Biotech) 20 mM Tris-HCl (pH 7.6), containing 50 mM KCl, 1 mM DTT, 1 mM $MgCl_2$, 0.2 mM EDTA, 0.01% Triton X-100, 5% glycerol, 0.5 mM spermidine, and 0.15 μg double-stranded poly (dI-dC). Mixtures loaded onto a 5% nondenaturing polyacrylamide gel were electrophoresed in a 0.4× Tris-Borate/EDTA buffer (pH 8.3), containing 36 mM Tris, 36 mM boric acid, and 0.8 mM EDTA, at 150 V (constant voltage) at 4°C for 3 h. The gel was then transferred to Whatman paper, vacuum-dried, exposed to Hyperfilm (Amersham Pharmacia Biotech) with an intensifier screen for 1 to 2 days, and then applied to a Molecular Imager FX (Bio-Rad) for digital imaging.

Oligonucleotides Used for EMSA—The following are the sequences of the upper-strands of oligonucleotides obtained from Hokkaido System Science (Sapporo) and used for EMSA. –65 to –41 bp of the normal fragment of the gp91^{phox} promoter (5′-CTGTTTTCATTTCCTCATTGGAAGA-3′) was used as the probe and the wild-type competitor. Some typical competitors were shown below. –57C mutant competitor, 5′-CTGTTTTCCTTTCCTCATTGGAAGA-3′; –56C mutant competitor, 5′-CTGTTTTCACTTCCTCATTGGAAGA-3′; –56G mutant competitor, 5′-CTGTTTTCAGTTCCTCATTGGAAGA-3′; –55C mutant competitor, 5′-CTGTTTTCATCTCCTCATTGGAAGA-3′, –52T mutant competitor, 5′-CTGTTTTCATTTCTTCATTGGAAGA-3′; –51G mutant competitor, 5′-CTGTTTTCATTTCCGCATTGGAAGA-3′; –50G mutant competitor, 5′-CTGTTTTCATTTCCTGATTGGAAGA-3′; –56C/–51G/–49T mutant competitor, 5′-CTGTTTTCACTTCCGCTTTGGAAGA-3′; –56C/–49T mutant competitor, 5′-CTGTTTTCACTTCCTCTTTGGAAGA-3′; –56C/–51G mutant competitor, 5′-CTGTTTTCACTCCGCATTGGAAGA-3′; –51G/–49T mutant competitor, 5′-CTGTTTTCATTTCCGCTTTGGAAGA-3′. The underlined end sequence represents mutated bases

Statistical Analyses—Data are presented as means ± one standard deviation (SD) and were usually obtained for three or more independent experiments performed in duplicate or triplicate. Statistical differences were determined by means of the paired Student's *t*-test and a *p* value of lower than 0.05 was taken to be significant.

RESULTS

Activities of gp91^{phox} Promoter Constructs Varied with Single-Point Mutations—We previously proposed PU.1 is an essential activator of the gp91^{phox} gene in human neutrophils, monocytes, and B-lymphocytes, based on the finding that the –50G mutation with selectively decreased binding to PU.1, but not the –56G mutation with selectively decreased binding to HAF-1, results in impaired promoter

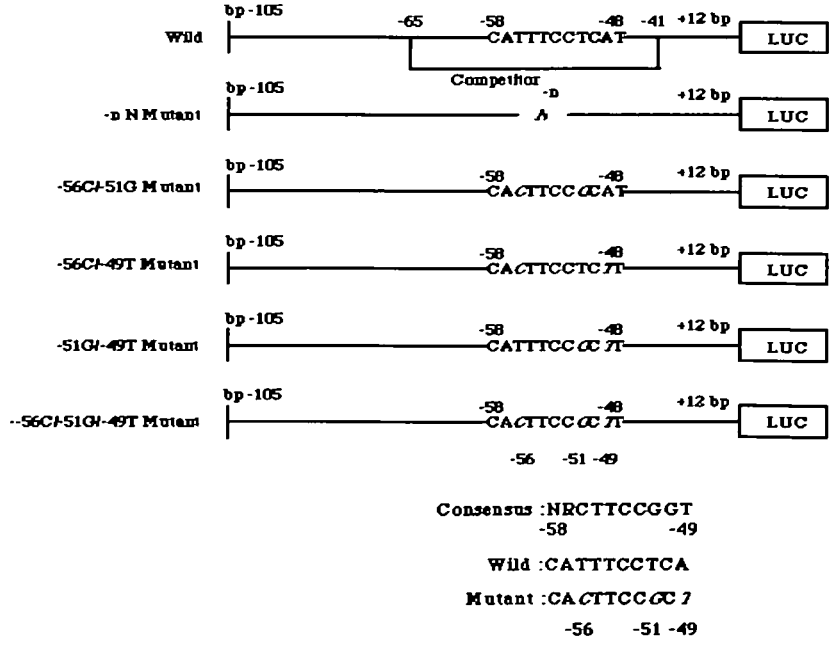

Fig 1 **Schematic representation of the wild-type and variously mutated constructs of the gp91^{phox} promoter.** The wild-type (Wild) and various other constructs of the gp91^{phox} promoter were linked to a luciferase reporter gene (LUC) and then transiently transfected into PLB-985 cells as described under "MATERIALS AND METHODS." Sequences derived from the gp91^{phox} gene are shown in capitals. Italics indicate the mutated sequences. In mutant –*n*N, the wild-type base at bp –*n* is changed to N. Mutated bases are shown by italics.

activity in erythroleukemic HEL cells (*14*). In our preliminary experiments on monocytically differentiated PLB-985 cells, however, the –50G-mutated gp91^{phox} promoter construct exhibited comparable promoter activity to the normal construct in PLB-985 cells, suggesting a significant role for HAF-1 in activation of the gp91^{phox} promoter in human myelogenous cells. Therefore, we attempted to elucidate the relative contributions of these two endogenous transcription factors upon activation of the human gp91^{phox} promoter in monocytically differentiated PLB-985 cells by using additional mutant constructs.

Using a wild-type gp91^{phox} –105 to +12 bp promoter construct (Wild) as a template, we prepared a series of promoter-luciferase reporter constructs containing single-point mutations at the bp *–n* site including the Pu box (*14*), and lying between bp –58 and –48 (*–n*N Mutant), as shown in Fig. 1. The promoter activities of all possible single-point-mutated constructs were first assayed as reported previously (*30*), and are shown in Fig. 2. It should be noted that TPA was used to potentiate the transfection efficiency and to monocytically differentiate PLB-985 cells (*30, 31*) as mentioned under "MATERIALS AND METHODS" Therefore, data presented hereafter reflect the differentiated state of PLB-985 cells. All four single-point mutants (–57C, –55C, –53T, and –52T) discovered in novel CGD patients (*14, 15*) exhibit dramatically low promoter activities. Because these CGD mutant activities have been demonstrated in PLB-985 cells that express no artificially introduced genes of transcription factors, the activities observed in our system reflect the *in vivo* activities of the gp91^{phox} promoter in the cells In 27 mutants, including those in the CGD patients, the promoter activity is significantly lower than that of the wild-type construct. The activity is significantly higher than that of the wild-type for the following single-point mutations, –56C, –51G, and –49T. These three individual base changes make the construct closer to the consensus sequence (–58NRCTTCCGGT–49, Fig 1, bottom) for the Ets protein (*24*)

Activities of Single-Point-Mutated gp91^{phox} Promoter Constructs Correlate with Their Binding Affinities to PU.1 But Not to HAF-1—Using the wild-type 25-mer sequence from bp –65 to –41 as a probe, competitions of these single-point-mutated 25-mer fragments have been quantitatively analysed by EMSA, as illustrated (Fig. 3A), using the wild-type

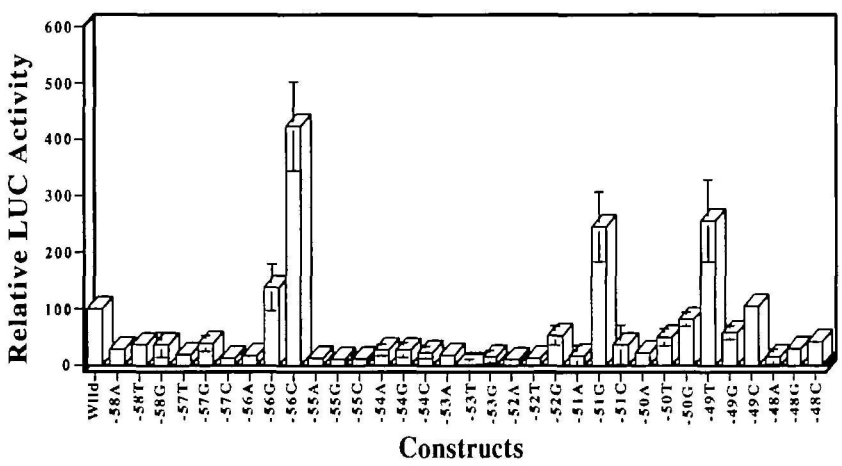

Fig 2 **Promoter activities of various constructs with single-point mutations at the Pu box of the human gp91^{phox} gene.** Each single-base mutant of the gp91^{phox} promoter linked to the firefly luciferase reporter plasmid shown in Fig 1 was transiently transfected into PLB-985 cells Relative firefly luciferase activities normalised by co-transfected renilla luciferase activities are shown as percentages assuming the wild-type (Wild) promoter activity to be 100% Each column and bar are the relative mean of three independent values and SD, respectively Mutations of –57C, –55C, –53T, and –52T have been reported in patients with X-linked CGD For more details, see "MATERIALS AND METHODS"

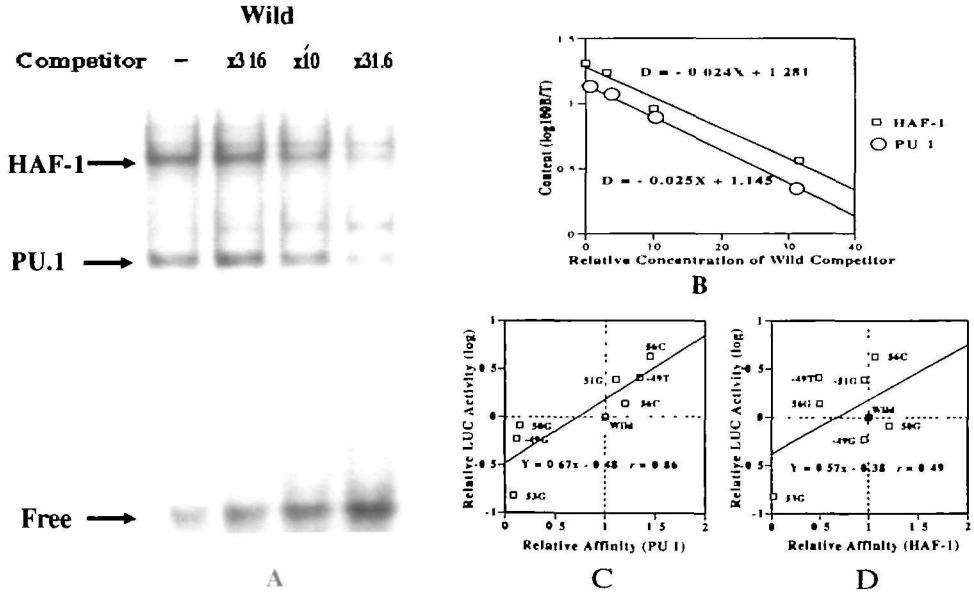

Fig 3 **Activities of the single-point-mutated gp91^{phox} promoter constructs correlate with their binding to PU.1 but not to HAF-1.** Competition on EMSA of a 3.16–31 6-fold molar excess of wild-type (Wild) competitor is illustrated in A. From these data, fitted slopes of plots for the relative concentration of the wild-type competitor to the probe concentration (*X*-axis) against the relative content of the probe in a band to each total amount of the probe (*Y*-axis) were obtained (B) The wild-type competitor (B) gives negative slopes for specific binding of PU1 (circles) and HAF-1 (squares) C and D show the correlation of the relative promoter activity of the wild-type and 7 *–n*N constructs with their binding affinities to PU 1 and HAF-1, respectively

sequence as the competitor. The logarithmic amounts of the probe [log (100 × shifted counts/total counts)] shifted by PU.1 or HAF-1 were plotted against the relative concentrations of a competitor (Fig. 3B). Each slope of the fitted line for each set of points was used as the value reflecting the binding affinity. Assuming the individual slopes for PU1 and HAF-1 obtained with the wild-type competitor to be 1, the relative affinities of all four mutants identified in CGD are presented in Table I. The relative affinities of all CGD mutants to PU.1 and HAF-1 are dramatically low, except for the affinity of the –57C mutant to HAF-1, which was about half that of the wild-type construct. Therefore, either PU.1 or HAF-1 should be essential for activation of the gp91^{phox} promoter in monocytically differentiated PLB-985 cells. However, HAF-1 may not be as efficient as PU.1, because the affinity of the –57C mutant (0.55) to HAF-1 is significantly high compared with the affinity of PU.1 to the –52T mutant, which exhibits the best affinity for PU.1 among the CGD mutants (0.20, $p < 0.05$).

In order to detect any quantitative relationship between the promoter activity and the binding affinity of PU.1 or HAF-1, we determined the relative affinities of eight constructs. These were the wild-type construct and 7 –*n*N mutants, comprising one CGD equivalent (–53G), three point mutants (–56G, –50G, and –49G) exhibiting moderate promoter activities, and three mutants exhibiting high promoter activities (–56C, –51G, and –49T). The relative affinities of these sequences to PU.1 (Fig. 3C) and HAF-1 (Fig 3D) were plotted against the corresponding logarithmic values of promoter activities. The CGD equivalent mutant (–53G, Fig. 2) also exhibits low affinity binding to both factors, again suggesting that either one is essential for activation of the gp91^{phox} promoter. Other mutated gp91^{phox} promoter activities essentially increase or decrease according to an increase or decrease, respectively, in the binding affinity to PU.1 (Fig. 3C). Constructs –49G and –50G, however, exhibited high promoter activities in spite of their low affinities to PU.1. Because the affinities of these two to HAF-1 were similar to or higher than that of the wild-type construct (Fig. 3D), HAF-1 may be able to compensate for the promoter activity otherwise decreased by the poor binding of PU.1. The positive correlation of PU.1 ($Y = 0.67X–0.48$, $r = 0.86$) is higher than that of HAF-1 ($Y = 0.57X–0.38$, $r = 0.49$). Therefore, we suggest that endogenous PU.1 binding

TABLE I **Promoter activities of CGD mutants, and their binding affinities to PU.1 and HAF-1.**

Construct	Relative LUC (%)	Activity (*n*)	Relative affinity* PU1	(*n*)	HAF-1	(*n*)
Wild	100 ± 0	(6)	1 0 ± 0	(10)	1 0 ± 0	(10)
–57C	12 6 ± 5 1	(3)	0 03 ± 0 13	(4)	0 55 ± 0 23	(4)
–55C	11 3 ± 7 3	(3)	–0 22 ± 0 14	(4)	0 14 ± 0 09	(4)
–53T	15 0 ± 10 0	(3)	–0 15 ± 0 26	(3)	–0 06 ± 0 08	(3)
–52T	13 0 ± 3 3	(3)	0 20 ± 0 12	(4)	0 01 ± 0 15	(4)

*Relative affinity was expressed as a (slope of individual competitor)/(slope of wild-type (Wild) competitor) ± SD *n* is the number of independent experiments performed for each mutant

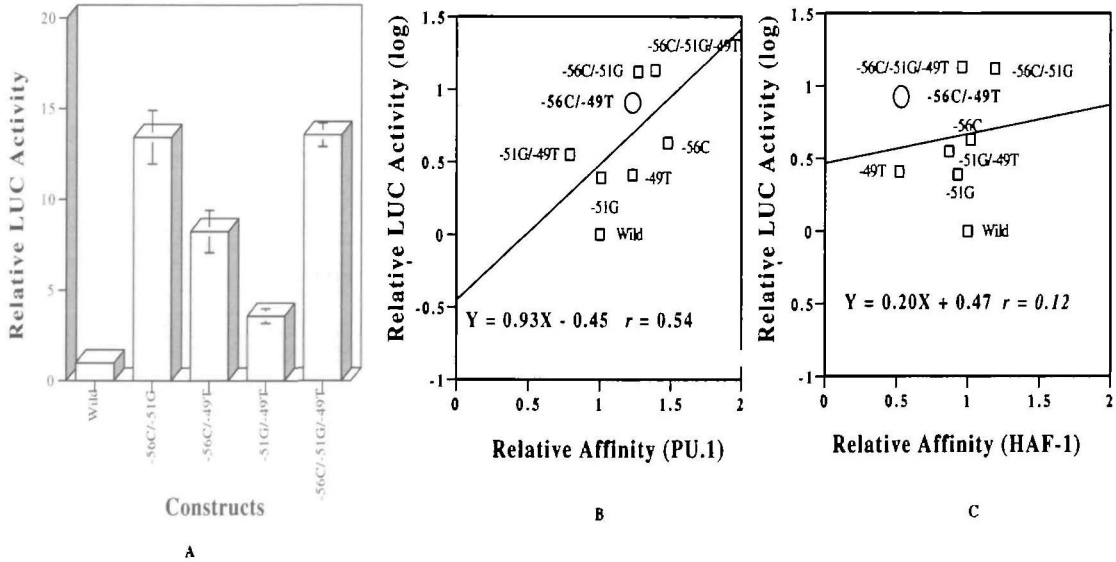

Fig 4 **Multiple-point mutants closer to the consensus sequence of the Ets-binding site exhibit high gp91^{phox} promoter activities accompanying high affinities to PU.1.** Three double-, and one triple-point-mutated (at bps –56, –51, and –49) gp91^{phox} promoter construct were linked to firefly luciferase-reporter plasmids and then transiently transfected into PLB-985 cells (A) Each column and bar are the relative mean of four independent values and SD, respectively, assuming the activity of the wild-type (Wild) construct to be 1 All four constructs exhibits 4- to 14-fold higher promoter activity than the wild-type. The relative promoter activities of these multiply mutated constructs, as well as all three single-point-mutated constructs and the wild-type (Fig 3), are plotted against the means of the three relative affinities of the corresponding constructs to PU1 (B) and HAF-1 (C) A circle indicates the point for the construct (–56C/–49T) used in the cotransfection experiment (Fig 5)

to the Pu box of the gp91^{phox} promoter dominantly activates it *in vivo* and that HAF-1 binding to the box is supplementary.

Increased Activities of Promoters with Closer Sequences to the Ets-Consensus Element Correlate with Their Binding Affinities to PU.1 but Not to HAF-1—If PU.1 is actually the dominant activator, promoter activity artificially exaggerated by certain mutations introduced to the Pu box should accompany exaggerated binding of PU.1, but not necessarily that of HAF-1. This possibility was examined with combinations of three single-point mutations (–56C, –51G, and –49T), each of which increased the gp91^{phox} promoter activity (Fig. 1). All constructs with the four possible combinations were confirmed to bind to PU 1 and HAF-1, and gave no additional bands on EMSA (data not shown). Therefore, the different promoter activities due to these constructs should be attributed to these two transactivators. Figure 4A clearly demonstrates that all four constructs exhibit 4- to 14-fold increased activity compared to that of the wild-type construct. The means of these promoter activities as well as those of the wild-type and the three single-point-mutated constructs were plotted against the means of their relative affinities to PU.1 and HAF-1 (Fig. 4, B and C). This figure, therefore, contains all possible combinations of the three mutations. There is significantly positive correlation between the promoter activities and binding affinities to PU.1 ($p < 0.025$, $Y = 0.93X–0.45$, $r = 0.54$, $n = 3 \times 8$), but not to HAF-1 ($Y = 0.20X + 0\ 47$, $r = 0.12$), supporting PU.1 is the dominant activator.

Co-Expressed Dominant-Negative PU.1 but Not Wild-Type PU.1 Decreases the Pu Box-Dependent Expression of the gp91^{phox} Gene—If PU.1 is the dominant activator of the gp91^{phox} promoter *in vivo*, the exogenously introduced dominant-negative PU.1 gene is expected to decrease the promoter activity of the gp91^{phox} gene. The dominant-negative gene was co-transfected into PLB-985 cells with the –56C/–49T promoter-reporter plasmid (Fig. 5). The promoter activity of the –56C/–49T construct was significantly low in the co-transfected cells compared with that of mock-transfected cells ($p < 0.001$). This low activity is specific because the promoter activity was significantly increased by the co-transfected wild-type PU.1 gene ($p < 0.005$). This result is consistent with PU.1 being the dominant transactivator of the gp91^{phox} gene in PLB-985 cells.

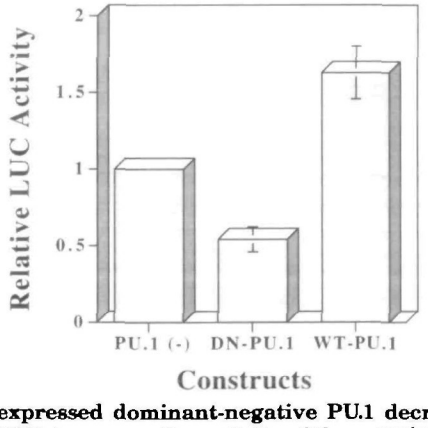

Fig. 5 **Co-expressed dominant-negative PU.1 decreases and wild-type PU.1 increases the activity of the gp91^{phox} promoter with a sequence optimised for PU.1 in PLB-985 cells.** A mock plasmid [PU.1 (–)] or an expression plasmid (0 4 μg) for either dominant-negative PU 1 (DN-PU 1) or wild-type (WT-PU 1) was co-transfected with the –56C/–49T promoter-firefly luciferase reporter plasmid (1 6 μg) into PLB-985 cells in three independent sets of experiments The net increase in the promoter activity of the mock co-transfected specimen [PU 1 (–)] was assumed to be 1

DISCUSSION

Higher promoter activity correlates with the higher affinity of PU.1 but not HAF-1 to the Pu box of the promoter of the gp91^{phox} gene in monocytically differentiated PLB-985 cells (Figs. 3 and 4). Transiently expressed dominant-negative PU.1 significantly decreases the promoter activity of the gene (Fig. 5). These results support the mechanism in which PU.1 binding to the Pu box activates dominantly the gp91^{phox} promoter in PLB-985 cells. Our results suggest that HAF-1 is still important as a supplementary activator because the significant promoter activity of mutants exhibiting deficient binding to PU.1 (–50G and –49G) can be attributed to the significant binding of these mutant constructs to HAF-1 (Fig. 3, C and D). Although previously reported gp91^{phox} promoter activities were mostly dependent on the products of exogenously introduced and over-expressed PU.1 or Elf-1 gene for HAF-1 (*25, 28*), the promoter activities presented here, except for those shown in the right hand column (WT-PU 1) of Fig. 5, are completely dependent on endogenous PU.1 and HAF-1. Therefore, our present results strongly suggest that endogenous PU.1 and HAF-1 actually activate the gp91^{phox} promoter *in vivo* in human monocytes/macrophages as dominant and supplementary transcription factors, respectively. Accordingly, the activities of both factors should be simultaneously decreased if bactericidal reactive oxygen species generated by monocytes/macrophages are harmful to hosts in inflammation, and PU.1 activity but not HAF-1 activity should primarily increase in the opposite situation.

We previously concluded that PU.1 is essential for expression of the human gp91^{phox} gene based on the results obtained for HEL cells (*14*) HAF-1, however, can partly substitute for PU 1 in monocytic PLB-985 cells. Although the mechanism underlying this gain-of-function in HAF-1 was not focussed on here, the contribution of this mechanism to activation of the gp91^{phox} promoter may be still limited. It should be noted that the promoter activation by highly co-expressed Elf-1-containing HAF-1 approached only about one-seventh of that achieved by highly co-expressed PU.1 (*25*).

It was interesting to find that PU.1, but not HAF-1, exhibits increased binding affinity to a series of point-mutated sequences closer to the consensus Ets-binding sequence, which is optimal and common to PU.1 and Elf-1 (*25–27*). The base requirement of Elf-1 for optimal binding might be altered in HAF-1 by other components such as IRF-1 and TF1^{phox} (*25, 28, 35, 36*). The optimal sequence for GATA-1 of a complex with PU.1 is reportedly different from that for GATA-1 in its single form (*37*).

Logarithmic values of the gp91^{phox} promoter activities linearly correlate with the binding affinities of PU.1 to its Pu box, indicating that the increased binding strength of PU.1 results in an exponential increase in transcription of the gene. A simple interaction between this transactivator and the Pu box still allows this phenomenon to occur because multiple functional domains in one molecule can give it

allosteric potential. The involvement of other factors such as IRF-4 and ICSBP is also possible, as in the case of the activation of human interleukin-1β (IL-1β) (*38*).

All four single-point-mutated promoters reported in CGD patients (*14, 15*) exhibit impaired promoter activity (Fig. 2), and are consistently deficient in binding to both PU.1 and HAF-1 (Fig. 3, C and D). Therefore, even other single-point mutations with deficient promoter activity (–57T, –55A, –55G, –53G, and –52A) may result in deficient binding of both factors, as exemplified by –53G (Fig. 3, C and D), and cause CGD. We predict that neutrophils, monocytes, and B-lymphocytes, but not eosinophils, of these possible CGD patients are deficient in the expression of gp91^{phox}, as reported for a –53T patient and a –52T patient (*14, 16*). The sequences optimised here for the maximal binding to PU.1 might be more useful than the wild-type sequence for gene therapy for X-linked CGD patients when physiologically controllable gene transfer is desired.

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