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GATA-3 represses gp91^{phox} gene expression in eosinophil-committed HL-60-C15 cells

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Abstract To study the regulatory mechanism of gp91^{phox} gene expression in eosinophils, we transiently transfected eosinophil-committed HL-60-C15 cells with gp91^{phox} promoter constructs, and identified a negative element from bp −267 to −246 of the gp91^{phox} gene, the deletion of which caused an 83% increase in promoter activity. Electrophoresis mobility shift assays demonstrated GATA-3 binds to the GATA consensus site from bp −256 to −250. An 81% increment in promoter activity was obtained when a mutation was introduced in the GATA-3 binding site of the bp −267 to +12 construct, which is comparable to that of the bp −245 to +12 construct. We therefore conclude that GATA-3 specifically binding to the GATA site negatively regulates the expression of the gene in HL-60-C15 cells.

Key words: gp91^{phox}; Eosinophil; Chronic granulomatous disease; GATA-3; GATA consensus site

1. Introduction

Eosinophils, like other professional phagocytes (neutrophils, monocytes and macrophages), are involved in host defense against invaders, especially parasites, by producing superoxide anion [1,2]. The cells are also involved in allergic responses and killing of tumor cells [3]. When the cells are challenged with a variety of stimuli, the phagocyte NADPH oxidase becomes rapidly active to generate reactive oxygen intermediates [4,5]. Any defects in a key component of NADPH oxidase, gp91^{phox}, lead in approximately two-thirds of cases to chronic granulomatous diseases (CGDs) [6].

The gp91^{phox} gene is exclusively expressed in phagocytic cells differentiated beyond the promyelocytic stage and B lymphocytes [7]. It has been reported that inflammatory mediators such as lipopolysaccharide, tumor necrosis factor α, granulocyte-macrophage colony-stimulating factor and interferon-γ (IFN-γ) augment the transcription of the gp91^{phox} gene [8,9]. To date, a number of regulatory *cis*-elements in the proximal promoter of the gp91^{phox} gene and *trans*-acting factors including IRF-1, IRF-2, CP1, BID, TF1^{phox} and PU.1 as activators, and CDP as a repressor have been identified in non-eosinophilic lineages [10–13]. We discovered a novel CGD patient with normal superoxide-generating activity in eosinophils [14,13], suggesting an eosinophil-specific regulatory mechanism for the gp91^{phox} gene. To understand the eosinophil-specific transcriptional mechanism for the gene, we used eosinophil-committed HL-60-C15 cells that express the gp91^{phox} gene, and identified a GATA binding site as a negative *cis*-element of the gp91^{phox} promoter and GATA-3 as a repressor. GATA-3 is expressed in eosinophil lineages but not in other phagocytic or B lymphocytic lineages [15,16]. Accordingly, the eosinophil lineage uses, at least in part, its own mechanisms for the inhibition of gp91^{phox} gene expression.

2. Materials and methods

2.1. Construction of plasmid for promoter analysis

Various deletive gp91^{phox} promoter constructs were prepared from a plasmid containing the human gp91^{phox} gene sequence from bp −487 to +12 inserted into a polycloning site of a promoterless luciferase vector pGVB2 (Toyoink, Tokyo) as follows. The plasmid was linearized by *Sac*I, and the 5′ overhang sequence was exposed by *Xho*I at its 5′ end to make the site sensitive to exonuclease III. The plasmid DNA extracted by phenol/chloroform was precipitated with isopropanol, and dissolved into 100 μl of exonuclease III buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol). 1 μl of exonuclease III (Takara, Japan) was added to the DNA solution and the mixture was immediately placed in a water bath incubator kept at 37°C. During the incubation with exonuclease III, an aliquot of 10 μl of the above solution was taken at 0.5 min and every 2 min thereafter until 20.5 min into an Eppendorf tube containing 10 μl of ice-cold MB nuclease buffer (40 mM Na-acetate, pH 4.5, 100 mM NaCl, 2 mM $ZnCl_2$, 10% glycerol) and immediately placed on dry ice. The mixture was incubated at 65°C for 5 min for the inactivation of the exonuclease III, cooled on ice for 2 min, and incubated with 10 units of MB nuclease at 37°C for 30 min to make blunt ends at both sides of linearized and digested plasmid DNA. The plasmid DNA was extracted, precipitated as above and resuspended in 9 μl of T4 DNA ligation buffer (50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 50 μg/ml BSA) to which 1 μl of T4 DNA ligase (New England BioLabs, Beverly, MA) was added and the mixture was incubated overnight at 16°C. The ligated plasmid was transformed into competent *Escherichia coli* DH5α which was spread onto carbenicillin agar plates. After overnight incubation each colony was expanded into 5 ml LB broth culture medium, from which plasmids were recovered and sequenced by a dideoxy termination method [17] using the AutoRead Sequencing Kit II (Pharmacia/LKB, Uppsala, Sweden) and ALF DNA Sequencer II (Pharmacia/LKB). Deletion mutants confirmed to contain the bp −301 to +12, bp −267 to +12, and bp −245 to +12 regions of the gp91^{phox} gene were subcloned into the *Kpn*I and *Nco*I sites of luciferase expressing pGVB2 basic vector.

The GATA mutant bp −267 to +12 luciferase reporter construct (Fig. 4) was made by PCR amplification of the gp91^{phox} promoter with a mutant sequence having the TC to AG substitution at bp −253 to −252 as a primer. The mutant construct was verified by DNA sequencing and subcloned into the *Kpn*I and *Nco*I site of a luciferase expressing pGVB2 vector.

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Abbreviations: CGD, chronic granulomatous disease; BID, binding increased during differentiation; INF-γ, interferon-γ; CDP, CCAAT displacement protein; IRF, interferon responsive factor; EMSA, electrophoretic mobility shift assay; TBP, TATA box binding protein; TCRβ, T-cell receptor β

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2.2. Cell culture

HL-60-C15, an eosinophil-committed subline of HL-60, a promyelocytic leukemia cell line, was a generous gift from S.A. Fischkoff [18]. The cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 2 mM L-glutamine (Wako Life Science, Tokyo, Japan), 26 mM $NaHCO_3$ (Wako Life Science) and passaged twice a week.

2.3. Transient transfections

HL-60-C15 cells were transiently transfected by electroporation using a Bio-Rad gene pulser II (Bio-Rad Laboratories, Hercules, CA) as follows. Cells (5×10^6) in 300 μl of HEPES-buffered (25 mM, pH 7.4), serum-free RPMI 1640 medium were put in a 4-mm gapped electroporation cuvette (Bio-Rad) containing 10 μg of gp91^{phox} promoter firefly luciferase plasmid and 0.05 μg of CMV promoter/enhancer renilla luciferase plasmid (pRL-CMV) (Promega, Madison, WI) as an internal control. The cells in the cuvette were gently pipetted for mixing, kept at room temperature for 15 min, and electroporated at 310 V with a capacitance of 950 μF. The cells were kept on ice for 15 min and grown in 8 ml of RPMI 1640 supplemented with 10% FBS (Hyclone) and 2 mM glutamine at 37°C under 5% CO_2-95% air. After 10 h of incubation, cells were washed twice with Ca^{2+}/Mg^{2+}-free phosphate-buffered saline, lysed in 100 μl of 1×PLB (Passive lysis buffer) (Promega) by a 10 s sonication on ice and allowed to lyse completely for 20 min. Solubilized cell lysates were collected by centrifugation at 21 000×*g* for 2 min and an aliquot of 10 μl lysate was used for simultaneous assays of the two luciferase activities using the Dual-Luciferase Reporter Assay System (Promega) and a luminometer, Berthold Multi-Biolumat LB 9505C (Berthold, Wildbad, Germany). The reporting activity of firefly luciferase was normalized by a simultaneously assayed renilla luciferase activity. Each mean value of the reporter activity was obtained from at least three independent triplicate values using at least two different preparations of plasmid DNA (Qiagen Plasmid Maxi Kit, Qiagen, Valencia, CA). Statistical significance was determined by Student's *t*-test.

2.4. Preparation of nuclear extracts

Nuclear extracts were prepared from culture cells essentially according to the method of Edgar Schreiber et al. [19] with minor modifications. Briefly, 10^7 cells in a suspension of 400 μl of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg leupeptin, 2 μg aprotinin, 10 μg E-64, 10 μg dichloroisocoumarin, and 10 μg pepstatin A (Sigma, St. Louis, MO)) was allowed to swell on ice for 15 min, 25 μl of a 10% solution of Nonidet NP-40 (Nacalai Tesque, Kyoto) was added to the suspension, which was vigorously vortexed for 10 s and centrifuged for 1 min (12 000×*g*, 4°C). The precipitate obtained was resuspended in 100 μl of cold buffer C (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 2 μg leupeptin, and 2 μg aprotinin), rotated for 30 min at 4°C, and centrifuged for 5 min (21 000×*g*, 4°C). Nuclear extracts recovered as supernatant were divided into aliquots (50 μl each) and immediately frozen to −80°C. Protein concentration was assayed spectroscopically using the Bio-Rad protein assay.

2.5. Electrophoretic mobility shift assay (EMSA)

Sense and antisense oligonucleotide (oligo) probes dissolved in 400 μl of annealing buffer (100 mM Tris-HCl, pH 8.0, 1.5 mM NaCl, 10 mM EDTA) were denatured at 100°C for 5 min and annealed at room temperature for 2 h. The duplex was labeled with [α-^{32}P]ddATP (3000 Ci/ml) using terminal deoxynucleotidyl transferase (TdT) (3′-END labeling kit, Amersham International, Amersham, UK). Five μg of nuclear extracts was incubated on ice for 15 min with or without one of 300-fold molar excess cold competitors in 20 μl of binding reaction mixture (20 mM Tris-HCl, pH 7.6, 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 0.5 μg double stranded poly(dI-dC)). ^{32}P-labeled DNA probe (1×10^4 cpm) was added to the reaction mixture, which was further incubated on ice for 15 min. The mixture was loaded on 3.4% polyacrylamide in 0.4×TBE buffer and electrophoresed at 4°C under a constant current of 25 mA. The gel was dried and analyzed with a GS-250 Molecular Image Analyzer (Bio-Rad).

Antibody supershift assay was performed using 4 μg of *mono*-specific polyclonal antibodies raised against the carboxy-terminal of either GATA-1, GATA-2 or GATA-3 (Santa Cruz Biotechnology, Santa Cruz, CA), or control goat IgG. Before addition of the radiolabeled probe, the nuclear extract was incubated with an antibody on ice for 1 h and electrophoresis was done as mentioned above.

Following are sequences of the upper strands of double stranded oligos used in EMSAs as labeled and unlabeled probes: oligo 1 (#1), bp −301 to −257 of gp91^{phox} promoter; 5′-AGTTATTTCACTGTGTAAAATACATCCCTTAAAATGCACTGTTAT-3′; #2, bp −260 to −240 of gp91^{phox} promoter, 5′-TTATTTATCTCTTAGTTGTAG-3′; #3, bp −250 to −230 of gp91^{phox} promoter, 5′-CTTAGTTGTAGAAATTGGTTT-3′; #4, bp −260 to −240 of gp91^{phox} promoter containing a two-point mutation (indicated in bold face), at a GATA consensus site, 5′-TTATTTA**AG**TCTTAGTTGTAG-3′. We used other oligos, one was the GATA consensus oligo from the human T-cell receptor δ gene [20] and the other its GATA-mutant one: #5, 5′-CACTTGATAACAGAAAGTGATAACTCT-3′, and #6, 5′-CACT-T**CT**TAACAGAAAGT**CT**TAA CTCT-3′ (mutated nucleotides shown in bold face).

3. Results and discussion

To determine the eosinophil-specific regulatory element in the human gp91^{phox} gene promoter region, eosinophil-committed HL-60-C15 cells were transiently transfected with a

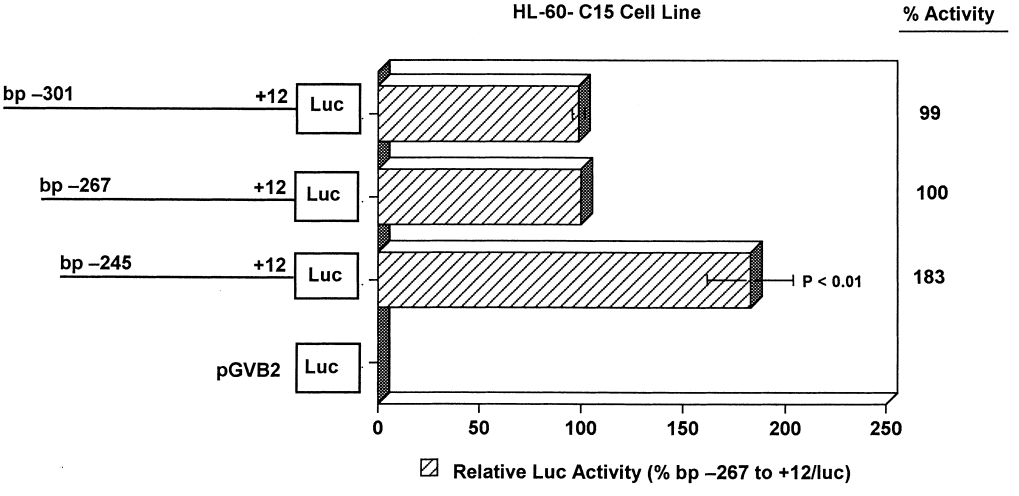

Fig. 1. Functional analysis of human gp91^{phox} promoter constructs in eosinophil-committed HL-60-C15 cells. 5′ deletion mutants of the gp91^{phox} promoter in the pGVB2 luciferase expression vector schematically shown on the left were transiently transfected into HL-60-C15 cells and reported luciferase activities were determined as described in Section 2. Bar is one S.E.M.

series of 5′-promoter deletion constructs. The deletion of the promoter sequence from bp −301 to −268 did not change any reporter activity (Fig. 1). But an 83% increment in promoter activity was obtained on further deletion down to bp −246. These results indicate the sequence from bp −267 to −246 of the gp91^{phox} gene is a negative element for the expression of the gp91^{phox} gene.

To determine a nuclear protein(s) binding to the negative element of the gp91^{phox} promoter, EMSAs were performed using nuclear protein extracts from HL-60-C15 cells. First, we used two overlapping gp91^{phox} oligo promoter fragments, #1 and #2, as labeled probes that covered the negative element (Fig. 2A). No specific DNA binding protein complexes were found when #1 (Fig. 2B) but #2 (Fig. 2C, lane 1, indicated by an arrow) was used as a probe. As computer analysis revealed the GATA consensus site (5′-TTATCTC-3′) centered at bp −253 (Fig. 2A), we used #3 having no GATA site and #5 derived from the T-cell receptor δ gene containing GATA consensus sequences [20] as competitors with the expectation that the specific DNA binding protein(s) might be GATAs (Fig. 2C, lanes 4 and 5 respectively). As shown in Fig. 2C, the DNA binding protein(s) was GATA(s) because the complex was definitely inhibited by an excess amount of the wild competitor, #2 (lane 2) and also by #5 (lane 5) but not by an excess amount of #1 (lane 3) or #3 (lane 4). Oligos #4 and #6, which are GATA-mutant versions of #2 and #5, respectively, failed to inhibit complex formation (lanes 6 and 7). On the other hand, if #4 was used as a probe, no complex

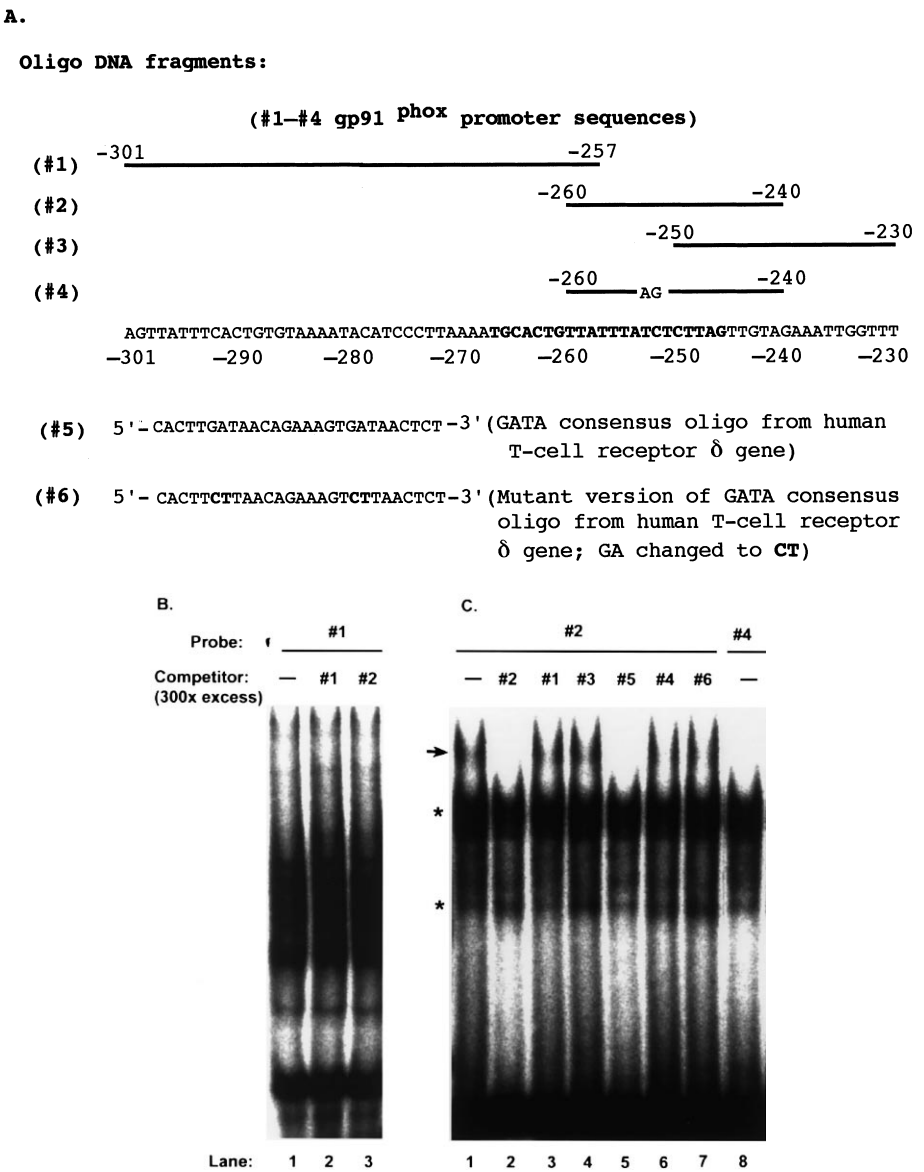

Fig. 2. Detection of DNA binding protein(s) specifically recognizing the negative element of the gp91^{phox} promoter (bp −267 to −246) in HL-60-C15 cells. A: Oligo DNA fragments (oligo) of the human gp91^{phox} promoter region (#1–#4) and GATA consensus oligos (#5 and #6) of the T-cell receptor δ gene are schematically shown. The negative element of the gp91^{phox} promoter region (data from Fig. 1) is in bold face. Oligo #4 is a GATA mutant of #2. B: EMSAs were performed using oligo #1 as a labeled probe and 5 μg of nuclear extracts from HL-60-C15 cells in the absence (lane 1) or presence of a molar excess of cold competitors (lane 2, #1; lane 3, #2) as described in Section 2. C: EMSAs were performed using #2 (lanes 1-7) or #4 (lane 8) as probes and the nuclear extracts of HL-60-C15 cells as in B in the absence (lanes 1, 8) or presence of one of the following fragments as a competitor: #2 (lane 2), #1 (lane 3), #3 (lane 4), #5 (lane 5), #4 (lane 6) and #6 (lane7). The arrow indicates the specific DNA-protein complex and the asterisks indicate non-specific complexes.

was found at the level of the specific complex formed by #2 (lane 8). Therefore, we speculated that the GATA consensus site centered at bp −253 is important for the negative regulation of the gp91^{phox} gene in HL-60-C15 cells, and the GATA binding protein(s) might be *trans*-acting factor(s).

To identify a nuclear protein(s) specifically binding to the GATA consensus site in HL-60-C15 cells, EMSAs were performed using bp −260 to −240 (#2) as the labeled probe and *mono*-specific antibodies against human GATA-1, GATA-2 and GATA-3 (Fig. 3). The GATA consensus sequence-specific complex is indicated by an arrow (lane 1). Neither supershift nor inhibition of the complex formation by anti-GATA-1 (lane 3) and anti-GATA-2 antibodies (lane 4) was observed as in the case of control goat IgG (lane 6). But the formation of the complex was almost completely inhibited by anti-GATA-3 antibody (lane 5). These results indicate that GATA-3, but not GATA-1 or GATA-2, binds to the GATA consensus site of the gp91^{phox} promoter in HL-60-C15 cells.

From the above data, we have shown in HL-60-C15 cells that GATA-3 is the protein that specifically binds to the GATA consensus site (bp −256 to −250) in the negative element of the gp91^{phox} gene. Therefore GATA-3 was supposed to be a repressor for the expression of the gene. To determine whether GATA-3 is actually a repressor or not, we transiently transfected the cells with either the wild bp −267 to +12 promoter construct or the GATA-mutant one in which the TC to AG substitution was introduced to the GATA consensus site because the substitution made #2 lose its binding activity to GATA-3 (Fig. 2C, lane 8). The mutant construct exhibited promoter activity 81% higher than the wild construct did (Fig. 4B, $P < 0.01$). The activity is comparable to that of the bp −245 to +12 construct (Fig. 1). These

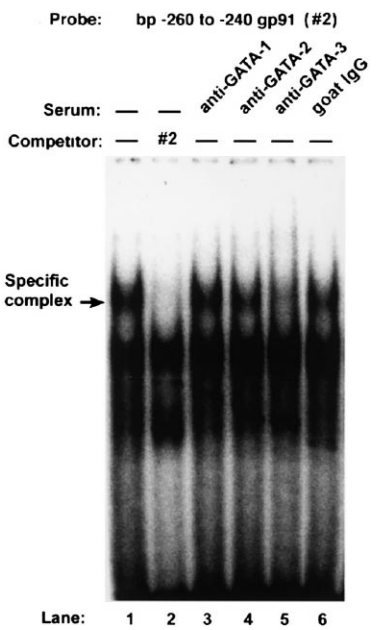

Fig. 3. Identification of protein in HL-60-C15 cells that binds to the GATA binding site in the human gp91^{phox} promoter. Using the bp −260 to −240 gp91^{phox} promoter fragment (#2) as a probe EMSA was performed as described in Section 2. Nuclear protein extracts from HL-60-C15 cells were preincubated with goat antibodies against GATAs. An arrow indicates a specific DNA-protein complex.

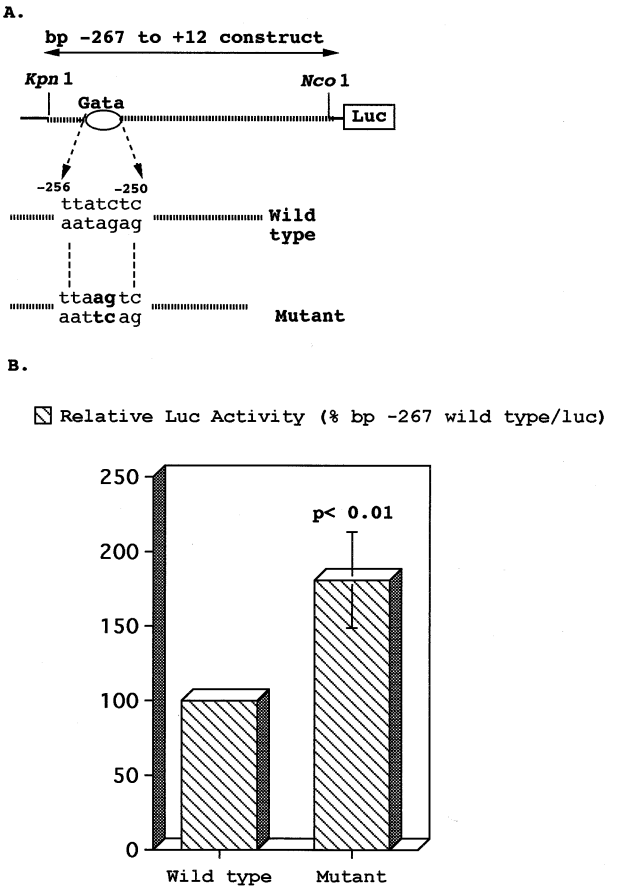

Fig. 4. Comparative activities of wild type gp91^{phox} promoter construct and its GATA-mutant version in HL-60-C15 cells. A: Wild type and GATA-mutant gp91^{phox} promoter luciferase constructs are schematically shown. Mutated AG are shown in bold face. B: The wild bp −267 to +12 pGVB2 firefly luciferase construct and its GATA-mutant form were transiently transfected in HL-60-C15 cells, and luciferase activities were detected as described in Section 2. Bar is one S.E.M.

results indicate that GATA-3 is the repressor for gp91^{phox} gene expression in eosinophilic HL-60-C15 cells.

To date, GATA-3 mRNA has been reported only in peripheral eosinophils but not in other gp91^{phox}-expressive cell lineages (neutrophils, macrophage or B lymphocytes) [15,16]. In this study, we have shown that GATA-3 expressed in eosinophil-committed HL-60-C15 cells binds to the gp91^{phox} promoter and represses gene expression. Therefore, GATA-3 acts as an eosinophil lineage-specific repressor of the gp91^{phox} gene. Though mRNAs for GATA-1 and GATA-2 are present in HL60-C15 cells [15], we could not detect these factors possibly because of too low contents of them to be detected by EMSA.

GATA-3 has been reported as a repressor in the human erythropoietin (Epo) gene [21] and the 17β-hydroxysteroid dehydrogenase type 1 gene [22]. Repression mechanisms of GATA-3 can be classified into two groups depending on the competition with TBP. In one mechanism, GATA-3 competes with TBP to bind at the GATA site lying in place of the TATA box in TATA-less genes [23]. In these cases, a GATA sequence, but not its inverted one, lies on an upper strand. In the other mechanism, GATA-3 binds to a GATA site lying far from the TATA box in the promoter, and in-

hibits gene expression without competing TBP. The latter mechanism has definitely been demonstrated in the 17β-hydroxysteroid dehydrogenase type 1 gene [22]. The GATA-3 repression mechanism for the human gp91^{phox} gene is consistent with the second one but not the first one because the gene has a TATA box, the GATA consensus site is inverted and located far from its core promoter.

A few other transcriptional factors have been proposed as repressors of the gp91^{phox} gene in non-eosinophilic myeloid cells. Human CDP binds to multiple sites in the promoter, competes with CP1, and is downregulated in the course of phagocytic differentiation allowing expression of the gene [24]. IRF-2 is supposed to bind to the site centered at bp −230 in competition with TF1^{phox}, an activator [12]. GATA-3 is unlikely to compete with other factors on the site centered at bp −253 in HL-60-C15 cells because other factors binding to the same site were not found.

In conclusion, we have identified GATA-3 as the first defined eosinophilic lineage-specific repressor of human gp91^{phox} gene. GATA-3 may regulate gp91^{phox} gene expression to an optimal level with a possible eosinophil-specific activator for sufficient killing of invading parasites and for avoiding unwanted toxic effects of reactive oxygen species. The GATA consensus site identified should be an ideal target for transient gene therapy for increasing eosinophil-specific expression of gp91^{phox} to achieve potential killing of parasites.

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