GATA Transcription Factors Regulate the Expression of the Human Eosinophil-derived Neurotoxin (RNase 2) Gene*

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The transcription factors GATA-1 and GATA-2 have been implicated in promoting differentiation of eosinophil leukocytes. In this study, we examined the roles of GATA-1 and GATA-2 in activating transcription of the secretory ribonuclease, the eosinophil-derived neurotoxin (EDN/RNase 2). Augmented expression of both GATA-1 and GATA-2 was detected in eosinophil promyelocyte HL-60 clone 15 cells in response to biochemical differentiation with butyric acid. Deletion or mutation of one or both of the two consensus GATA-binding sites in the extended 1000-bp 5′ promoter of the EDN gene resulted in profound reduction in reporter gene activity. Antibody-augmented electrophoretic mobility shift and chromatin immunoprecipitation analyses indicate that GATA-1 and GATA-2 proteins bind to both functional GATA consensus sequences in the EDN promoter. Interestingly, RNA silencing of GATA-1 alone had no impact on EDN expression; silencing of GATA-2 resulted in diminished expression of EDN, and also diminished expression of GATA-1 in both butyric acid-induced HL-60 clone 15 cells and in differentiating human eosinophils derived from CD34+ hematopoietic progenitors. Likewise, overexpression of GATA-2 in uninduced HL-60 clone 15 cells resulted in augmented transcription of both EDN and GATA-1. Taken together, our data suggest that GATA-2 functions directly via interactions with the EDN promoter and also indirectly, via its ability to regulate the expression of GATA-1 in differentiating eosinophils and eosinophil cell lines.

Eosinophils remain among the most enigmatic of the mammalian leukocytes, although consensus opinion is that they contribute in some fashion to the pathophysiology of allergic asthma and gastrointestinal dysfunction (1). Despite disagreement as to their function, eosinophils are clearly identified in peripheral blood and tissues by their unique morphology and staining characteristics, including their characteristic bilobed nuclei and large refractile granules containing distinct, cationic secretory proteins.

Eosinophils differentiate from pluripotent stem cells in bone marrow, and they can be induced to develop from CD34 antigen-positive progenitor cells in vitro in response to cytokine stimulation (2). Whereas no unique events have been identified that define the eosinophil lineage specifically, transcription factors that contribute in various ways to eosinophil development include C/EBPα, C/EBPε, PU.1, and GATA-1 and -2, although the precise temporal and kinetic interplay between these factors remains to be elucidated (3, 4). The recent finding that ablation of a palindromic dbGATA enhancer-binding site in the promoter of the mouse Gata-1 gene results in eosinophil-lineage ablation in vivo (5) has resulted in a particular focus on the role of GATA transcription factors in promoting eosinophil development and differentiation. GATA-1 was first described as promoting transcription of erythroid genes (reviewed in Refs. 6–8), and Zon et al. (9) were the first to demonstrate expression of the hematopoietic GATA factors (GATA-1, GATA-2, and GATA-3) in isolated human eosinophils and eosinophilic cell lines. Kulessa et al. (10) found that overexpression of GATA-1 converts avian myelomonocytic cell lines into other lineages, including eosinophils, and Hirasa et al. (11) discovered the same to be true for human primary myeloid progenitors, with GATA-2 compensating effectively for GATA-1 both in vitro and in vivo. Similarly, Iwasaki et al. (12) reported that GATA-2 overexpression in C/EBPα-expressing granulocyte/monocyte progenitor cultures likewise resulted in eosinophil commitment and differentiation. At the molecular level, functional GATA-1 enhancer sites direct the expression of the eosinophil granule major basic protein (13), and GATA-1 directs the eosinophil-specific expression of gp91phox, with GATA-2 functioning as a site-specific repressor (14).

Here we examine the role of GATA transcription factors in directing the expression of the human eosinophil secretory ribonuclease, the eosinophil-derived neurotoxin (EDN/RNase 2). EDN² is a rapidly evolving RNase A ribonuclease with several intriguing biological activities (reviewed in Ref. 15). EDN was first identified as a neurotoxin (16, 17), as microgram quantities of this protein injected intrathecally into experimental animals resulted in Purkinje cell loss and concomitant ataxia. EDN also has antiviral properties (reviewed in Ref. 18), and recent studies suggest that EDN interacts with dendritic cells via signaling through the toll-like receptor TLR2 (19).

In an initial study utilizing minimal functional promoters, we determined that full transcriptional activity of EDN required the presence of one or more intron-based enhancer elements.

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2 The abbreviations used are: EDN, eosinophil-derived neurotoxin; RNase, ribonuclease; siRNA, silencing RNA; HEPPSO, N-2-hydroxyethylpiperazine-N-2-hydroxypropanesulfonic acid; RT, reverse transcription; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; BA, butyric acid; IL, interleukin; shRNA, short hairpin RNA; EMSA, electrophoretic mobility shift assay.
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(20, 21). In subsequent studies, de Groot and co-workers (22, 23) demonstrated that both PU.1 and C/EBP consensus sites were crucial for EDN expression, and recently Wang et al. (24) identified regulatory elements in a 34-bp proximal promoter segment shared among primate EDNs. In this study, we focus on the extended 1000-bp 5’ promoter of EDN and utilize RNA silencing methods to explore the specific roles of GATA-1 and GATA-2 transcription factors in promoting its expression.

EXPERIMENTAL PROCEDURES

Preparation of Reporter Constructs—Human genomic DNA (Clontech) and DNA purified from clone BAC R-84C10 (Bacpac Resources, Oakland, CA) were used as templates to amplify extended regions of genomic sequence 5’ to the known transcriptional start site of the EDN gene (20, 25). The 5’ regions were amplified by PCR in a 50-μl reaction containing 5 μl of 10× buffer, 1.5 μl of 10 mM dNTP, 1 μl of 50 mM MgSO₄, 1.5 μl of 10 μM forward primer, 1.5 μl of 10 μM reverse primer, and 1.0 μl (1.25 units) of Pfx polymerase (Invitrogen). Reaction conditions were 94 °C for 2 min, followed by 35 cycles of 94 °C for 15 s, 55 °C for 30 s, and 68 °C for 3 min. Primers were as follows: EDN 1000 forward, 5’-CGG TAC CTT CCC TAA AGT CCC TGA AAA CTC C-3’; EDN 500 forward, 5’-CGG TAC CTA ATT ATC TAC AGA ATC TTG TGC CCC-3’; EDN 250 forward, 5’-CGG TAC CGC ATA TAG TTT TCA TCC AGA GTT T-3’; and EDN all reverse, 5’-GCT CGA GCT GTA AGA AAA GAA GAG AAG TAA C-3’. KpnI and XhoI restriction sites were added to the ends of both forward and reverse primers, respectively, to facilitate cloning. All the PCR products were gel-purified using GFX PCR DNA and Gel band purification kit (Amersham Biosciences Biotech). A terminal nucleotide was added with the A-Addition kit (Qiagen), and the insert was ligated into the T/A cloning vector (Invitrogen), followed by transformation into TOPO 10 Escherichia coli (Invitrogen). Inserts were verified by sequencing, re-isolated by KpnI and XhoI digestion, and cloned into pGL3 Basic reporter vectors (Promega, Madison, WI).

Cell Culture—The HL-60 clone 15 human eosinophil promyelocytic leukemia cell line (26), purchased from American Type Culture Collection (Manassas, VA), was cultured in RPMI 1640 (Invitrogen) supplemented with 10% fetal calf serum, 1% penicillin and streptomycin (Invitrogen), 1% L-glutamine (Invitrogen), 5% CO₂ in a humidified incubator. Unless otherwise indicated, cells were differentiated towards an eosinophilic phenotype for 2 days prior to transfection.

Transient Transfection Experiments—Culture medium as described was pre-warmed at 37 °C, 5% carbon dioxide prior to harvesting for luciferase assay. Western Blotting—HL-60 clone 15 cells differentiated for 2 days with 0.5 mM BA or uninduced cells transfected with GATA-2/pcDNA3.1 or pcDNA3.1 vector only (control) as described below were centrifuged at 8000 rpm for 5 min, and the pellet was washed with PBS and resuspended in lysis buffer (25 mM Tris, pH 8.0, 0.5% Triton X) plus 0.1% 4-(2-aminoethyl)benzenesulfonyl fluoride (ICN Biomedicals, Inc., Eschwege, Germany). The cells were lysed and heated for 5 min after adding the identical volume of 2X Tris-glycine SDS Sample Buffer (Invitrogen). The proteins were resolved in 14% Tris-glycine gel (Invitrogen) and transferred onto nitrocellulose membrane filter paper (Invitrogen). After blocking nonspecific protein binding with 5% nonfat dry milk, blots were incubated with 1:1000 dilutions of monoclonal antibodies to EDN (MBL, Naka-Ku, Japan), GATA-1 (Santa Cruz Biotechnology, Santa Cruz, CA), and 1.0 μl of Luciferase Assay Reagent II (LARII; Promega) was placed into luminometer tubes, and 20 μl of cell lystate was added and pipetted three times prior to reading firefly (FF) luciferase activity. Immediately, 100 μl of Stop & Glo Reagent (Promega) was added and vortexed, and then Renilla luciferase (RL) activity was read. All measurements were repeated three times for each data point. Data are reported as relative light units (FF/RL × 100).

Site-directed Mutagenesis of Consensus GATA-binding Sites—The primers for introducing mutations into the identified consensus GATA-binding sites in the extended 5’ promoter region of the EDN gene were designed based on sequence analysis results with the MatInspector program available online. The primers for site-directed mutagenesis of GATA sites include the following: 5’-CTG AAA ACT CCC TGC GGC GAT TTT AGG GCC CTG CTG-3’ and 5’-CAG CAG GAG GCC CCT AAA CTC GGC GCA GGG AGT TTT CAG-3’ for the EDN-1144 site; 5’-AGC AGA TTG TTT TAA GGC GAG ACA GAA TCT TGT GCC-3’ and 5’-GCG ACA AGA TTT TTC GGT CTC GCC TTA AAA CAA TCT GCT-3’ for the EDN-535 site. Mutant strand synthesis reaction was performed in 50 μl of mixture containing 50 ng of DNA template, 125 ng of each primer, and 2.5 units of Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA) at 95 °C for 30 s and 18 cycles of 95 °C for 30 s, 55 °C for 1 min, and 68 °C for 6 min. After the parental double-stranded DNA was digested with DpnI for 1 h at 37 °C, the synthesis products were transformed into XL1-Blue E. coli Supercompetent cells (Stratagene), and then the desired mutations were verified using DNA sequencing (3100 Sequencer, Applied Biosystems).

Dual Luciferase Assays—The transiently transfected cells described above were harvested by centrifugation at 5000 rpm for 5 min. The cell pellets were washed with once with cold phosphate-buffered saline (PBS) and resuspended in 80 μl of 1× passive lysis buffer (PLB; Promega). The incubation was performed on a rocking platform at room temperature for 15 min, and then luciferase activity was measured using a TD20/20 luminometer (Turner Designs, Sunnyvale, CA). Briefly, 100 μl of luciferase assay reagent II (LARII; Promega) was placed into luminometer tubes, and 20 μl of cell lystate was added and pipetted three times prior to reading firefly (FF) luciferase activity. Immediately, 100 μl of Stop & Glo Reagent (Promega) was added and vortexed, and then Renilla luciferase (RL) activity was read. All measurements were repeated three times for each data point. Data are reported as relative light units (FF/RL × 100).
or GAPDH (US Biological, Swampscott, MA) or GATA-2-specific polyclonal antibody (Santa Cruz Biotechnology) followed by alkaline phosphatase-conjugated goat anti-mouse, -rat, and -rabbit IgG (Bio-Rad). Immunoprecipitates were detected with alkaline phosphatase color-developing reagents (Bio-Rad) following the manufacturer’s instructions.

Quantitative RT-PCR—The cells treated as described were homogenized in RNA-Bee RNA Isolation Solvent (at 1 ml per 10⁶ cells, increased five times over the manufacturer’s specifications to accommodate increased ribonuclease activity; TelTest, Inc., Friendswood, TX). The total RNA was prepared following the manufacturer’s instruction, and its quality was monitored through gel electrophoresis analysis. Genomic DNA contamination was eliminated with DNA-free™ DNase treatment and removal reagent (Ambion, Austin, TX). RNA was reverse-transcribed in a mixture of 20 µl containing 5 mM MgCl₂, 1 mM dNTP, 1.6 µg of oligo-(dT)₁₂, 50 µl of RNase inhibitor, and 20 units of avian myeloblastosis virus reverse transcriptase (Roche Applied Science) at 25°C for 10 min, 42°C for 1 h, and 99°C for 5 min. After incubation at 50°C for 2 min and 95°C for 10 min, quantitative PCR was performed in 7500 real-time PCR system (Applied Biosystems) for 40 cycles at 95°C for 15 s and 60°C for 1 min using Taqman® 2× universal PCR master mix (Applied Biosystems). The primers for EDN, GATA-1, and GATA-2 were purchased from Applied Biosystems. GATA-2 assay identification was Hs00927739_m1, and probe was 5'-GCC GCT GCC TGT GCC CGT TGC C-3'; EDN assay identification was Hs0795553_s1, and probe was 5’-CCC AGC GCT GCC AGA ACC AC-3'; GAPDH expression was identified with Hs00231112_m1, and probe was 5’-CAC CAG CCC TTA ATC CCC AGA G-3'; the probes were labeled with FAM and NFQ. GAPDH expression assay part number was 4326317E used as the endogenous control (VIC/MGB probe).

Nuclear Extract Preparation—HL-60 Clone 15 cells were different with 0.5 mM BA for 2 days, and 36% formaldehyde (Sigma) was added to a final concentration of 1% to cross-link proteins to DNA. Incubation was performed on a stir plate for 10 min at room temperature, and then the cross-linking reaction was stopped using Glycine Stop-Fix Solution as per manufacturer’s instructions (Active Motif). The cells were pelleted by centrifugation for 10 min at 2500 rpm at 4°C, washed with ice-cold PBS, and suspended in 1 ml of ice-cold Lysis Buffer supplemented with 5 µl of protease inhibitor mixture and 5 µl of 100 mM phenylmethylsulfonyl fluoride. Incubation was performed on ice for 30 min followed by homogenization in an ice-cold Dounce homogenizer. Pelleted nuclei were resuspended in 1.0 ml of Digestion Buffer supplemented with protease inhibitor mixture and phenylmethylsulfonyl fluoride as above; 50 µl of Enzymatic Shearing Mixture (200 units/ml; Active Motif) was added to shear the chromatin for 10 min. The sheared DNA was diluted 10-fold in Dilution buffer (Upstate; catalog number 20-153) and pre-cleaned by adding 60 µl of protein G-agarose (Upstate) in 1 h of incubation at 4°C with rotation, followed by removing agarose by a centrifugation of 4000 x g. Ten µl of pre-cleaned DNA was saved as the input template (cross-linked DNA, and the other was used in the immunoprecipitation reaction. For the negative control, 5 µg of preimmune rabbit IgG or mouse IgG was added; the test antibodies included 10 µg of rabbit anti-human GATA-1 anti-peptide antibody (Active Motif) or mouse anti-human GATA-2 antibody (Santa Cruz Biotechnology). Incubations were performed overnight at 4°C with rotation. 60 µl of protein G-agarose was added to the chromatin-protein with antibody mixture and incubated together for 1 h at 4°C. The protein G-agarose-antibody/chromatin complex was pelleted, washed with Low Salt Immune Complex wash buffer, High Low Salt Immune Complex wash buffer, LiCl Immune Complex wash buffer, and TE Buffer (Upstate). Protein-DNA complex was eluted from protein G-agarose with 200 µl of elution buffer (10 µl 20% SDS, 20 µl of 1 M NaHCO₃ and 170 µl of H₂O). For all samples, including input, negative control and experimental reverse cross-linking of protein/DNA was performed to isolate free DNA. Specifically, for each sample, 8 µl of 5 M NaCl was
added, and incubation was performed at 65 °C for 5 h, and then 1 μl of RNase A was added. After incubation at 37 °C for 30 min, 4 μl of 0.5 M EDTA, 8 μl of 1 M Tris-HCl, and 1 μl of proteinase K were added, and incubation was performed at 45 °C for 2 h. The resulting DNA was purified using spin columns (Upstate), and PCR was performed on a 50-μl sample containing 2 μl of DNA prepared as above, 5 μl of 10× buffer, 2 μl of 10 mM dNTP, and 0.5 μl of DNA polymerase. For the −1114 consensus GATA site in the EDN promoter, forward primer is 5′-CTGTGCCCAAGATGCTCATC-3′ and reverse primer is 5′-GCTTGAGGACAAGGACT-3′; for the −535 consensus GATA site, the forward primer is 5′-AGTGGATCCAATGCAAGG-3′ and reverse primer is 5′-TGAGCTATGATTCTTAGGGCACA-3′. PCR conditions were 3 min of 94 °C followed by 34 cycles of 20 s of 94 °C, 30 s of 58 °C, and 30 s of 72 °C.

RNA Silencing (siRNA)—The siRNA sequences commercially obtained from Ambion were 5′-GGUACUCAGUGCACCACUTT-3′ (GATA-1 siRNA), 5′-GGCCUGUUCUUCAGGAATT-3′ (GATA-2 siRNA 1), 5′-GGAGGAUGUGUGUUGATTT-3′ (GATA-2 siRNA 2). Negative control was Silencer Negative Control 1 siRNA (Ambion catalog number 4611). Three μg of siRNA was transfected into 2 × 10⁶ clone 15 cells in 100 μl of Nucleofector Solution V (Amaxa) with T-016 program using Nucleofector II machine (Amaxa) as described above. The cells were then differentiated with BA and harvested 48 h later, and the total RNA was isolated with RNA-Bee RNA Isolation Solvent (1 ml per 10⁶ cells) (Tel-Test, Inc). Quantitative PCR was used to analyze the extent to which levels of transcripts were reduced, and expression of protein encoding GATA-1, GATA-2, and EDN was evaluated by Western blotting.

GATA-2 Overexpression—cDNA encoding human GATA-2 (GenBank™ NM_032638) was isolated...
from BA-induced HL-60 clone 15 cells by RT-PCR using Pfx polymerase, forward primer 5'-CGG AAG CTT GCC GCC GGC CAT GGA GGT GGC-3' and reverse primer 5'-GGT TCT AGA CTA CCC CAT GGC GGT CAC CA-3', which includes restriction sites and a Kozak sequence at the start of translation. The insert was cloned into the HindIII/XbaI sites of pcDNA3.1/Zeo and confirmed by DNA sequencing. Three µg of GATA-2/pCDNA3.1 (pGATA-2) or pCDNA3.1 vector alone (pctrl) were transfected into 2 x 10^6 uninduced HL-60 clone 15 cells in 100 µl of Nucleofector solution V (Amaxa), T-016 program, Nucleofector II. Cells were resuspended in 2 ml of complete medium, and after 2 days, cells were harvested, and RNA was prepared using the qPCR-Grade RNA isolation kit (SuperArray Bioscience), and quantitative RT-PCR was performed as described.

**Culture and Differentiation of Human Umbilical Cord and Peripheral Blood CD34 Cells**—Human umbilical cord CD34 hematopoietic progenitors (Stemcell Technologies, catalog number CB008F) or CD34 progenitors from peripheral blood from healthy human donors pre-stimulated with granulocyte colony stimulating factor (a generous gift from Dr. Arnold Kirshenbaum, MCBS, LAD (26)) were cultured at 0.3 x 10^6 cells per ml in Iscove’s modified Dulbecco’s medium (Invitrogen) with 10% fetal bovine serum, 50 M mercaptoethanol, 2 mM glutamine, 10 units/ml penicillin, 10 units/ml streptomycin (basic medium), and cytokines, including stem cell factor (50 ng/ml), FLT-3L (50 ng/ml), granulocyte-macrophage colony-stimulating factor (5 ng/ml), IL-3 (5 ng/ml), and IL-5 (5 ng/ml, all cytokines from R&D Systems), as per Bedi et al. (27). After 3 days in culture, the medium was changed, and cells were maintained in the basic medium with cytokines IL-3 (5 ng/ml) and IL-5 (5 ng/ml) alone, at 0.5 x 10^6 cells/ml for the remainder of the experiment. Eosinophil differentiation over 21 days was monitored by modified Giemsa staining of cytocentrifuge cell preparations and EDN transcript levels.

**Suppression of GATA-2 Transcription in Eosinophil Progenitors Derived from CD34 Cells**—Recombinant lentiviral particles delivering shRNA to suppress GATA-2 transcription together with puromycin resistance were assembled in HEK293 cells as per the manufacturer’s instructions (Sigma Mission shRNA, with control plasmid pLK0.1 or with GATA-2 shRNA). 5 x 10^6 differentiating cells were transduced at day 14 of culture; 2 days later, fresh medium
with cytokines IL-3 (5 ng/ml) and IL-5 (5 ng/ml) was supplemented with puromycin (2 μg/ml, the optimal concentration determined by serial dilution, as per manufacturer’s instructions). Cells were harvested 5 days later for quantitative RT-PCR.

Statistical Considerations—All luciferase and quantitative PCR data represent compilations from three separate experiments with each data point from each experiment representing a trial performed in triplicate. Statistical significance determined by Student’s t test and Mann-Whitney U test as appropriate.

RESULTS

Expression of EDN, GATA-1, and GATA-2 in the HL-60 Clone 15 Eosinophil Promyelocyte Cell Line—The clone 15 subline of the HL-60 promyelocytic leukemia cell line has properties of an eosinophil progenitor when maintained under alkaline conditions. Addition of BA results in differentiation into cells with features characteristic of maturing human eosinophils (28, 29). We observe a 25-fold increase in EDN mRNA in response to BA at day 3 (Fig. 1A), consistent with previous results (9, 28). BA-induced differentiation of HL-60 clone 15 cells also results in augmented transcription of GATA-1 (Fig. 1B) and GATA-2 (Fig. 1C), reaching levels 50- and 20-fold over base line, respectively, at day 3. The GATA-2 expression pattern differs from that observed by Zon et al. (9) who performed Northern analysis and reported increased expression of GATA-1 but stable expression of GATA-2 in this cell line in response to 2 days of BA-induced differentiation. EDN, GATA-1, and GATA-2 proteins are not detectable at base line but are detected after 24 h of BA-induced differentiation (Fig. 1D). The indistinct banding pattern for EDN results from the heavy glycosylation that we described previously in this cell line (28).

Identification of GATA Consensus Binding Sites in the 5′ Promoter Regions of the Gene Encoding EDN—We isolated a 1435-bp fragment of the EDN gene that extended the characterized minimal 5′ functional promoter (20) and also included exon 1, the single intron, and a fragment of exon 2 proximal to the ATG translational start site. There are two GATA consensus binding sites in the extended 5′ promoter region, one at a distal position (bp −1114) and another at a more proximal site (bp −535). Luciferase reporter constructs, including the entire 1435-bp fragment described above (EDN 1000) as well as serial 5′ truncations (EDN 500 and EDN 250), are shown in Fig. 2A. Promoter activities of the EDN reporter constructs were evaluated in HL-60 clone 15 cells induced for 48 h with BA prior to transfection (Fig. 2B). The activity of the EDN 1000 construct was
nearly 50-fold over control (pGL3 basic reporter alone, no promoter included). The luciferase activities of the truncated EDN 500 and EDN 250 reporter constructs, with both GATA consensus sites eliminated, were reduced to ~20-fold over control (p < 0.05 versus EDN 1000). Similarly, site-specific mutagenesis of the distal GATA consensus binding site (−1114) of the EDN 1000 construct reduced the promoter activity more than 3-fold; a similar reduction was observed upon introduction of mutations into the proximal GATA site (−535), but no further reduction was observed with the double mutation (Fig. 2C). Of note, the activity of the extended 5′ promoter (EDN 1000) was not elevated in uninduced HL-60 clone 15 cells and was indistinguishable from that of the truncated EDN 250 and from that of EDN 1000 with both GATA sites mutated (EDN 1000 mGATA −1114/−535; Fig. 2D).

**GATA-1 and GATA-2 Binding to Consensus Sites in the EDN Promoter**—EMSA were performed with biotin-labeled probes that include the GATA sites within the EDN 5′ promoter (Fig. 3). We observed specific binding of proteins isolated from the BA-differentiated HL-60 clone 15 nuclear extract to the labeled probe that includes the GATA −1114 site (Fig. 3A, lane 2) but not to a probe with mutations within the core consensus GATA sequence (lane 5) nor in the presence of a 100-fold excess of unlabeled competing probe (lane 3). However, specific binding was detected in the presence of 100-fold excess unlabeled probe containing mutations (Fig. 3A, lane 4). Proteins specifically bound to the GATA −1114 probe were “super-shifted” by monoclonal antibody to GATA-1 (Fig. 3A, lane 6) and by polyclonal anti-GATA-2 (Fig. 3B, lane 8), indicating that both GATA-1 and GATA-2 proteins present in the nuclear extract can bind to this consensus site. The prominent band detected between the shift and supershift in Fig. 3A indicates separation between nonadjacent lanes.

**Chromatin Immunoprecipitation Analysis**—Formaldehyde-cross-linked protein-DNA complexes were isolated from BA-
differentiated HL-60 clone 15 cells, sheared, and subjected to immunoprecipitation with anti-GATA-1, anti-GATA-2, or control antibodies. Precipitated protein-DNA complexes were treated with proteases and used as a template to amplify consensus GATA-binding sites within the EDN promoter. Both GATA-1 and GATA-2 were detected in association with the distal −1114 GATA consensus binding site (Fig. 4A) and likewise with the proximal −535 GATA site (Fig. 4B); no amplification was observed when precipitation was attempted with control antibody.

**RNA Silencing of GATA-1 and GATA-2 Expression**—HL-60 clone 15 cells were transfected with a GATA-1-specific siRNA (GATA-1 siRNA) or an irrelevant control sequence as described under “Experimental Procedures.” Transfected cells were then differentiated with BA and harvested 48 h later, and RNA and protein were isolated and evaluated. As shown, transcription of GATA-1 was suppressed by 80% when compared with control levels (Fig. 5A). Interestingly, suppression of GATA-1 transcription and concomitant reduction of GATA-1

![Image](image_url)

**FIGURE 6.** Silencing of GATA-2 and its impact on EDN expression. A, transcription of GATA-2 in BA-differentiated HL-60 clone 15 cells transfected with control (irrelevant sequence) or one of two independent GATA-2-directed oligonucleotides was evaluated by quantitative RT-PCR. B, transcription of EDN was determined in the cells described in A. C, extracts prepared from the cells described in A were subjected to Western blotting and probed with anti-GATA-2, anti-EDN, or anti-GAPDH (loading control). Statistical significance is as follows: *, p < 0.05.

**FIGURE 7.** A, silencing of GATA-1 and its impact on GATA-2 expression; B, silencing of GATA-2 and its impact on GATA-1 expression. Transcription of GATA-1 or GATA-2 in HL-60 clone 15 cells transfected with irrelevant control (Ctrl) or one of two independent GATA-1- or GATA-2-directed oligonucleotides was evaluated by quantitative RT-PCR. Statistical significance is as follows: *, p < 0.01.

**FIGURE 8.** Expression of EDN and GATA-1 in response to overexpression of GATA-2. Relative expression of GATA-2 in uninduced HL-60 clone 15 cells 2 days after transduction with vector only (pctrl) or with the GATA-2 expression vector (pGATA-2) was evaluated by quantitative RT-PCR (A) and by Western blotting (B). Relative expression of EDN (C) and GATA-1 (D) in uninduced HL-60 clone 15 cells transduced as described was determined by quantitative RT-PCR. Statistical significance is as follows: *, p < 0.05; **, p < 0.01.
protein synthesis had a small but not statistically significant effect on the expression of EDN transcript (Fig. 5B) and minimal impact on expression of EDN protein (Fig. 5C). In contrast, transfection with two independent GATA-2-specific siRNAs resulted in suppression of GATA-2 transcription by ~70% when compared with control levels (Fig. 6A) and profound suppression of GATA-2 protein synthesis (Fig. 6C), accompanied by significant suppression of EDN (Fig. 6, B and C).

Given the critical role of GATA-2 in promoting expression of EDN, and findings suggesting that GATA-2 and GATA-1 have the potential to regulate one another’s expression (30–32), we explored the relative expression of GATA-1 under conditions of GATA-2 suppression and vice versa. Here we find that suppression of GATA-1 had no impact on the expression of GATA-2 (Fig. 7A), but suppression of GATA-2 has a profound impact on the transcription of GATA-1, reducing transcription to less than 10% of levels observed at base line (Fig. 7B).

**Overexpression of GATA-2**—Transfection of uninduced HL-60 clone 15 cells with the expression vector pcDNA3.1/GATA-2 (pGATA-2) results in prominent expression of GATA-2 over background levels (Fig. 8, A and B). Consistent with the RNA silencing findings, expression of GATA-2 has a significant impact on the expression of both EDN and of GATA-1; elevated levels of transcript of both EDN (Fig. 8C) and GATA-1 (Fig. 8D) were detected in cells overexpressing GATA-2 versus cells transfected with the control vector (pctlr).

**Suppression of GATA-2** in CD34⁺-derived Human Eosinophil Progenitors—CD34⁺ hematopoietic stem cells can be differentiated into eosinophilic progenitors using a specific cytokine regimen (“Experimental Procedures”) (27). The phenotypic progression of these cells is shown in Fig. 9A. By day 14, >70% of the cells have developed red-staining cytoplasmic granules, a hallmark of the eosinophilic promyelocyte; by day 21, the granulation has become more dense and intense, and many of the cells have developed rudimentary eccentric bilobed nuclei.

Lentivirus shRNAs were delivered to differentiating eosinophil cultures on day 14, and puromycin selection (2 μg/ml) was introduced on day 16, and cells were harvested for analysis on day 21. Under these conditions, expression of GATA-2 was reduced to ~15–20% of control levels (Fig. 9B); of note, puromycin selection resulted in no differential impact on cell number or percentage eosinophils, determined phenotypically, in the control or GATA-2-suppressed cultures (data not shown). In these same cultures, EDN expression was reduced to 50% of control (Fig. 9C), which was similar to the extent to which EDN expression was diminished in response to suppression of GATA-2 in the BA-induced clone 15 cells. GATA-1 expression was also reduced in response to GATA-2 suppression (Fig. 9D), although interesting, not nearly as profoundly as in the HL-60 clone 15 cells, only by about 25% when compared with the control. The reason for this differential response remains unclear.

**DISCUSSION**

In this work, we evaluated the contribution of two consensus GATA-binding sites to the activity of the extended functional promoters of EDN and eosinophil cationic protein. Interestingly, both GATA consensus sites are crucial for full promoter activity of EDN in BA-induced cells of the eosinophil promyelocyte clone 15 HL-60 cell line. We detect binding of both GATA-1 and GATA-2 proteins to consensus sites in the EDN promoter, but silencing of GATA-1 alone had little to no impact on EDN transcription or translation, possibly due to compensation from GATA-2, which binds to the same consensus sites as GATA-1 both in tissue culture and in vivo. Interestingly, we found that silencing of GATA-2 reduced EDN transcription, but also reduced transcription of GATA-1 in both the HL-60 clone 15 eosinophil promyelocyte cell line and in differentiating eosinophils derived from human CD34⁺ hematopoietic progenitors. As such, our results are consistent with a more complex mechanism, one that requires coordinated actions of both GATA factors at one or more consensus binding sites.

GATA-1 and GATA-2 are members of a larger family of GATA transcription factors and co-factors (reviewed in Refs.
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33–36), and although there is only limited amino acid sequence between the two proteins overall (~52%), both bind to DNA sequences within the uniquely flanked internal GATA motif A/T (GATA) A/G (37). Initially explored vis à vis their role in promoting erythroid development, both GATA-1 and GATA-2 have been implicated in eosinophil hematopoiesis. Most of the recent focus has been on GATA-1 given the finding that ablation of a dblGATA enhancer in the mouse GATA-1 promoter, a site that has been implicated in autoregulation of the GATA-1 promoter (38), leads to selective eosinophil ablation in vivo (5), although this blockade can be circumvented in vitro (39). The human and mouse GATA-1 promoters have substantial sequence homology (40, 41), and the palindromic dblGATA consensus sequence is fully conserved. However, it is not at all clear how this consensus site functions within the human GATA-1 promoter, and whether it is similarly indispensable for eosinophil hematopoiesis in vivo.

Several earlier studies have addressed the transcriptional regulation of EDN. Our group has explored a minimal promoter-exon-intron reporter construct, which was 2–3-fold more active in BA-differentiated clone 15 cells than in undifferentiated counterparts, and featured active enhancer elements in the intron (20, 21). de Groot and co-workers (22, 23) have characterized the roles of PU.1 and C/EBP in promoting EDN transcription, and most recently Chang and co-workers (24) have shown that MAX and Sp1 interact with the 34-bp segment unique to the proximal promoter region of EDN. Interestingly, given issues of evolutionary divergence, it will be difficult to evaluate the role of any of these factors in promoting transcription of EDN via gene-deletion experiments in vivo. EDN and ECP are rapidly evolving genes that are unique to primate species (42, 43); the mouse eosinophil-associated RNases are a cluster of 10–15 related genes, but there is no one specific mouse ortholog of EDN (44). We have characterized a fragment of an active promoter of mEar2 (45), one of the two eosinophil ribonuclease orthologs that are expressed prominently in mouse eosinophils (46), and for the purposes of this work, we have identified an extended 5′ promoter region from the mouse genome data base. This extended 5′ promoter sequence has no substantial homology to the 5′ promoter of EDN (<50%), but it does include three consensus GATA sites that might be the subject of future exploration.

In our study, which features gene transcription in both a differentiating eosinophil cell line and in human CD34+ progenitor cells cultured in eosinophilopoietic cytokines, we find that GATA-2 regulates not only a crucial eosinophil granule protein gene but also regulates the transcription of GATA-1. To the best of our knowledge, this is the first study in which promoter expression is evaluated in the presence of silencing RNAs directed against GATA-1 and GATA-2. Among the previous studies on eosinophil hematopoiesis that have focused on GATA-2, Iwasaki et al. (12) found that GATA-2 overexpression in C/EBPα-expressing granulocyte/monocyte progenitors resulted in eosinophil differentiation, whereas expression of GATA-1 under these conditions resulted in commitment toward the megakaryocytic lineage. At the same time, GATA-1-dependent expression of the gp91phox gene in eosinophils is suppressed by GATA-2 (14), which is quite different from what is observed with respect to GATA-2 and EDN.

Consensus opinion is clear that eliminating eosinophils is a viable and meaningful approach toward the amelioration of symptoms of allergic disease (reviewed in Ref. 47). Given the disappointing results with agents directed at eliminating eosinophils via anti-IL-5 cytokine modulation (48, 49), attention may turn toward transcriptional control as a more direct means of eliciting temporary eosinophil lineage ablation. No one has successfully identified a single transcription factor or transcriptional event that uniquely defines the eosinophil lineage, although GATA factors are likely to play a pivotal role. Ongoing studies may identify unique, eosinophil-specific mediators that interact directly with GATA factors during hematopoietic differentiation.

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