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The Crucial Role of GATA-1 in CCR3 Gene Transcription: Modulated Balance by Multiple GATA Elements in the CCR3 Regulatory Region

Byung Soo Kim,* Tae Gi Uhm,* Seol Kyoung Lee,* Sin-Hwa Lee,† Jin Hyun Kang,* Choon-Sik Park,† and Il Yup Chung*

GATA-1, a zinc finger-containing transcription factor, regulates not only the differentiation of eosinophils but also the expression of many eosinophil-specific genes. In the current study, we dissected CCR3 gene expression at the molecular level using several cell types that express varying levels of GATA-1 and CCR3. Chromatin immunoprecipitation analysis revealed that GATA-1 preferentially bound to sequences in both exon 1 and its proximal intron 1. A reporter plasmid assay showed that constructs harboring exon 1 and/or intron 1 sequences retained transactivation activity, which was essentially proportional to cellular levels of endogenous GATA-1. Introduction of a dominant-negative GATA-1 or small interfering RNA of GATA-1 resulted in a decrease in transcription activity of the CCR3 reporter. Both point mutation and EMSA analyses demonstrated that although GATA-1 exhibited a high binding affinity for GATA-1 and was solely responsible for GATA-1-mediated transactivation. The fourth and fifth GATA sites in exon 1, which were postulated previously to be a canonical double-GATA site for GATA-1-mediated transcription of eosinophil-specific genes, appeared to play an inhibitory role in transactivation, albeit with a high affinity for GATA-1. Furthermore, mutation of the seventh GATA site (present in intron 1) increased transcription, suggesting an inhibitory role. These data suggest that GATA-1 controls CCR3 transcription by interacting dynamically with the multiple GATA sites in the regulatory region of the CCR3 gene. The Journal of Immunology, 2010, 185: 000–000.

Eosinophils are granulocytes that are implicated in a wide variety of allergic diseases, including bronchial asthma. Eosinophils appear to be key effector cells in allergic diseases such as asthma; there is a positive correlation between increased numbers and activation of eosinophils and the severity of allergic symptoms (1, 2). Eosinophils develop in bone marrow, exit to the bloodstream, and enter inflamed tissues in response to proinflammatory mediators. Their transfer to the blood and tissues is attributed largely to CCR3 activity (3, 4). CCR3, an essential surface marker for eosinophils, is a major chemokine receptor that is strongly, if not exclusively, expressed on eosinophils and that, in concert with IL-5R, mediates eosinophil trafficking in the body (5, 6). Targeted disruption of the genes that encode CCR3 and/or its ligand eotaxin has been shown to reduce the recruitment of eosinophils to the blood and airways after Ag challenge in animal models of asthma (7–10). Likewise, administration of anti-CCR3 or anti-eotaxin Abs significantly reduces the numbers of eosinophils in the airways and circulation (11, 12). Several small molecule antagonists of CCR3 have been developed to inhibit eosinophil accumulation and airway hyperresponsiveness (13, 14).

A recent study revealed that CCR3 is expressed, along with IL-5Rα, in eosinophil progenitors at a very early stage in human eosinophil development (15). Hence, analysis of the transcription factors that control CCR3 expression may offer insights into the mechanisms behind the commitment of common myeloid progenitors to the eosinophil lineage. The CCR3 gene (Mendelian Inheritance in Man No. 601268) is located on chromosome 3p21.3 and consists of at least four exons (16). The critical sequence that accounts for transcriptional regulation of CCR3 has been mapped to exon 1 and its flanking sequences (16–19). Several lines of evidence have demonstrated unequivocally that GATA-1 plays an integral role in eosinophil development and the regulation of eosinophil-specific genes. Disruption of a high-affinity double-palindromic GATA site in the murine GATA-1 promoter results in the selective loss of the eosinophil lineage in vivo (20). GATA-1–transduced human CD34+ hematopoietic progenitor cells exclusively give rise to eosinophil lineage cells in vitro, whereas GATA-1–deficient mice fail to develop eosinophil progenitors in the fetal liver (21). GATA-1 reprograms avian myeloblastic cell lines to eosinophils, and its expression level fine-tunes development of the eosinophil lineage (22, 23). GATA-1 is expressed in human peripheral eosinophils and eosinophilic cell lines (24), and eosinophil progenitors are found exclusively within cells activating GATA-1 transcription (25). The promoters of the genes that encode the eosinophil-specific major basic proteins (MBPs), Charcot-Leyden crystal protein and eosinophil-derived neurotoxin...
through staining with Diff-Quick solution (Sysmex, Kobe, Japan). This basis of granule formation, and the shapes of the nuclei were visualized as 80% of the total cell population, as determined morphologically on the basis of granule formation, and the shapes of the nuclei were visualized.

Half of the cells were omitted from culture from days 7 and 14 onwards, respectively. The remaining half of the cells were supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), and DTT (5 mM). Reverse transcription was performed at 42°C for 1 h and followed by heat inactivation at 70°C for 15 min. Thus-synthesized cDNA was amplified for 30 cycles with Ex Taq DNA polymerase (TAKARA, Shiga, Japan). The following primers were used in the amplification: CCR3 forward primer 5'-ATGGGATGGTAAAGCTTCTC-TCAG-3' and CCR3 reverse primer 5'-TGCTGCCTCTACAGTCTTCC-CC-3'; GATA-1 forward primer 5'-GCCCTGACTTCTTTTCTAGTACC-3' and GATA-1 reverse primer 5'-CGAGTCTGATAACCATCCTCC-3'; GAPDH forward primer 5'-CGTCTACACCAGTGAAGA-3' and GAPDH reverse primer 5'-CGGCCATACGCGCAGATT3'.

Flow cytometry

Cells were fixed in 4% paraformaldehyde (Sigma-Aldrich) for 15 min at 4°C, washed with 2% BSA in PBS, and then stained with a PE-conjugated anti-human CCR3 Ab (R&D Systems, Minneapolis, MN) or isotype-matched Ab (BD Pharmingen). Fluorescence staining was analyzed using a FACSCalibur flow cytometer in conjunction with CellQuest software (Becton Dickinson, San Jose, CA).

Western blot analysis

Cells were lysed in RIPA buffer (50 mM Tris-Cl [pH 7.4], 0.1% NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM Na3VO4, 1 mM NaF, and protease inhibitor mixture [Calbiochem, San Diego, CA]) supplemented with 0.4 M NaCl. Lysates were centrifuged, and the resulting supernatants were subjected to Western blot analysis. Thirty micrograms of the cell lysates were resolved by SDS-PAGE and then transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat dry milk. Blots were probed with anti-GATA (H-200; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-GAPDH Abs (Santa Cruz Biotechnology) and then with an anti-rabbit HRP-conjugated Ab (Cell Signaling Technology, Beverly, MA). Immunostained proteins were detected using an ECL detection system (Amersham Biosciences).

ChIP analysis

Physical associations between GATA-1 and the CCR3 regulatory region in A549 and K562 cells were analyzed using a ChIP assay kit (Millipore, Bedford, MA), according to the manufacturer’s instructions. Cells were treated with formaldehyde for 10 min at 37°C, incubated in lysis buffer, and sonicated to shear the chromatin. The cross-linked protein–DNA complexes were immunoprecipitated with specific Abs raised against GATA-1 and sonicated to shear the chromatin. The cross-linked protein–DNA complexes were immunoprecipitated with specific Abs raised against GATA-1 (N6; Santa Cruz Biotechnology), histone 3 dimethyl K9, histone 3 tri-methyl K9, and histone 3 acetyl K9 (Abcam, Cambridge, U.K.). Rat IgG (Sigma-Aldrich) was used as a negative control for GATA-1, and an anti-histone 3 Ab (Abcam) was used as a positive control. After the Ab-bound complexes were pulled down with protein A-agarose/salmon sperm DNA (Millipore), the cross-links were reversed. DNA was recovered through phenol/chloroform extraction and ethanol precipitation and used as a template for PCR amplification. The sequences of the regulatory region of the CCR3 gene analyzed in the ChIP assay are shown in Fig. 1A, and the primers used in the amplification are listed in Supplemental Table I.

Reporter gene plasmids, mutagenesis, and luciferase assay

Regions of exon 1 and/or proximal intron 1 of the CCR3 gene shown in Fig. 1 were PCR-amplified and cloned into the pGL3-Basic luciferase reporter vector (Promega, Madison, WI). The PCR primers that were used are listed in Supplemental Table I. Mutation of GATA sequences was performed through conventional overlap extension PCR. All 5'-GATA-3'-sequences in exon 1 and proximal intron 1 of the CCR3 gene were mutated to 5'-TCGC-3'. Amplification was performed using four primers (including two overlapping primers for each mutation) and Pyrobest Taq (TAKARA) in a 50 μl reaction. The resulting PCR products were cloned into a pGL3 vector and their sequences were confirmed by sequence analysis. The primers used in the overlap extension PCR are listed in Supplemental Table I. Cell lines were transfected with the reporter plasmids using Lipofectamine 2000 reagent (Invitrogen Life Technologies). pSV-β-galac-

Materials and Methods

Cell culture

Human lung epithelial A549 and NCI-H292 cells and K562 cells were maintained in RPMI 1640 medium (Welgene, Seoul, Korea), and human embryonic kidney 293T cells were maintained in DMEM (Welgene). All of the growth media were supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). K562 cells differentiated in the presence of 0.5 mM butyric acid (BA; Sigma-Aldrich, St. Louis, MO). IL-5 (10 ng/ml) was then added on day 1. Thereafter, K562 cells were incubated for up to 7 d. Peripheral blood eosinophils were isolated from atopic individuals. After RBCs had been sedimented in 6% dextran–dextrose in 0.1 M EDTA (pH 7.4), the leukocyte-rich cell suspension was layered on Percoll solution (1.070 g/ml) and centrifuged at 400 × g for 20 min at 4°C. Enriched eosinophil fractions were incubated with anti-CD16 mAb-conjugated microbeads (Miltenyi Biotec, Auburn, CA), and contaminating neutrophils were removed through negative selection using a magnetic-activated cell sorter (BD Pharmingen, San Diego, CA). Mononuclear cells were isolated from umbilical cord blood as described previously (34) and plated at a density of 1 × 10⁶ cells per milliliter in IMDM (Welgene) supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), and HEPES (25 mM). Cells were cultured initially with GM-CSF (10 ng/ml), IL-3 (10 ng/ml), and IL-5 (10 ng/ml). GM-CSF and IL-3 were omitted from culture from days 7 and 14 onwards, respectively. Half of the culture medium was replaced weekly with fresh medium enriched with the appropriate cytokines. By culture day 28, eosinophilic cells comprised 40–80% of the total cell population, as determined morphologically on the basis of granule formation, and the shapes of the nuclei were visualized through staining with Diff-Quick solution (Sysmex, Kobe, Japan). This study was approved by the Soonchunhyang University Hospital Institutional Review Board (Protocol SCHBC-IRB-06-04).

CCR3 and GATA-1 mRNA expression

Total mRNA was extracted from four different cell lines, peripheral blood eosinophils, and umbilical cord blood-derived eosinophils using TRI reagent (Molecular Research Center, Cincinnati, OH). First-strand cDNA was synthesized from 4 μg total RNA using SuperScript II RNase Reverse Transcriptase (Invitrogen Life Technologies) in a 20 μl reaction including the random primers, deoxynucleotide triphosphates (0.5 mM), MgCl2 (2.5 mM), and DTT (5 mM). Reverse transcription was performed at 42°C for 1 h and followed by heat inactivation at 70°C for 15 min. Thus-synthesized cDNA was amplified for 30 cycles with Ex Taq DNA polymerase (TAKARA, Shiga, Japan). The following primers were used in the amplification: CCR3 forward primer 5'-ATGGGATGGTAAAGCTTCTC-TCAG-3' and CCR3 reverse primer 5'-TGCTGCCTCTACAGTCTTCC-CC-3'; GATA-1 forward primer 5'-GCCCTGACTTCTTTTCTAGTACC-3' and GATA-1 reverse primer 5'-CGAGTCTGATAACCATCCTCC-3'; GAPDH forward primer 5'-CGTCTACACCAGTGAAGA-3' and GAPDH reverse primer 5'-CGGCCATACGCGCAGATT3'.
tosidase (Promega) was cotransfected as an internal control. Cells were lysed 36 h posttransfection. Lysate luciferase activity was measured using the Luciferase Assay System (Promega), whereas β-galactosidase activity was assessed, through ortho-nitrophenyl-o-galactopyranoside hydrolysis, using a β-Galactosidase Enzyme Assay System Kit (Promega). Variations in transfection efficiency were corrected through normalization to β-galacto-
sidase activity. K562 cells were cotransfected with dominant-negative GATA-1 (DN-GATA-1), which was described previously (21), to probe the involvement of GATA-1 in reporter transactivation.

RNA interference
Small interfering RNA (siRNA)-dependent knockdown experiments were conducted in K562 cells using ON-TARGETplus SMARTpool siRNA GATA-1 and nontargeting (scrambled) control pools (Dharmacon, Chicago, IL). Briefly, K562 cells were cotransfected with siRNA and the reporter plasmid (Ex1-In1) using DharmaFECT Duo Transfection Reagent (Dharmacon). Thirty-six hours after transfection, luciferase activities were measured. An aliquot of the transfected cells was taken for later GATA-1 protein level analysis by immunoblot.

Nuclear extract preparation and EMSA
Nuclei obtained by centrifugation after lysis with RIPA buffer containing 0.15 M NaCl were resuspended in RIPA buffer containing 0.4 M NaCl. Lysates were centrifuged, and the resulting supernatants (nuclear extracts) were analyzed by EMSA. Four micrograms of the nuclear extracts were incubated with 32P-labeled probe for 20 min in binding buffer (50 mM Tris-Cl [pH 7.5], 20% glycerol, 5 mM MgCl2, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, and 0.25 mg/ml poly(dI-dC)poly(dI-dC)). For supershift analysis, extracts were incubated with anti–GATA-1 (M-20; Santa Cruz Biotechnology), anti–GATA-2 (Santa Cruz Biotechnology), anti-PU.1 (Santa Cruz Biotechnology), acute myeloid leukemia (AML)-1a (Santa Cruz Biotechnology), or control Abs (Sigma-Aldrich), followed by radiolabeled probe. Reaction mixtures were analyzed through separation on 4% polyacrylamide gels. The wild-type and mutant probe sequences that were used are listed in Supplemental Table IV.

Statistical analysis
Statistical analyses were performed using Microsoft Excel and SPSS, version 13.0 (SPSS, Chicago, IL). For in vitro analyses, differences between independent groups and samples were evaluated using a nonparametric Kruskal-Wallis test and corresponding post hoc analysis (18). Furthermore, introduction of GATA-1 siRNA led to a decrease in transcription activity of the CCR3 gene by a multiplex PCR, peaked on culture day 21, declining slightly thereafter. GATA-1 protein was not detectable in fresh mononuclear cells obtained from umbilical cord blood but was induced steadily as differentiation progressed (Fig. 2C). Therefore, it may be concluded that CCR3 expression generally parallels the expression of GATA-1, although these two genes are not always expressed coincidentally, as shown by the data from the K562 cells.

Results
Expression of CCR3 and GATA-1 mRNA and protein in cell lines, developing eosinophils, and peripheral blood eosinophils
GATA-1 is a key player in both eosinophil development (20, 21, 35) and the regulation of CCR3 expression (3, 19). We tested whether there was any correlation between the levels of expression of the genes encoding these two proteins. It has been shown that human lung epithelial A549 and NCI-H292 cells express CCR3 mRNA and protein (36, 37), whereas A549 and K562 erythroleukemia cells express CCR3 mRNA and protein (38, 39). Notably, K562 cells express high levels of GATA-1. We found CCR3 mRNA and protein to be modestly produced in human epithelial cells, including A549, NCI-H292, and 293T cells (Fig. 2A). In contrast, CCR3 expression was barely detectable in K562 cells but became evident after induction of differentiation Peripheral blood eosino-
nphils, as expected, constitutively expressed CCR3 mRNA and protein. All four cell lines analyzed expressed GATA-1 mRNA and protein with the following rank order: K562 > 293T > A549 > NCI-H292 (Fig. 2B). GATA-1 protein was not detectable in peripheral blood eosinophils, whereas its mRNA was barely detectable, the former being consistent with a previous finding (39). Next, we examined CCR3 and GATA-1 expression during the differentiation of umbilical cord blood-derived eosinophils. As we reported previously (34), CCR3 mRNA and protein levels gradually increased during the 28 d of culture. The GATA-1 mRNA level,
the CCR3 reporter with a concomitant reduction of GATA-1 protein levels in K562 cells in a dose-dependent manner, whereas scrambled siRNA had no effect (Fig. 4D). These data suggest that GATA-1 is a key transactivator of the CCR3 gene and that the sequences involved in regulation by GATA-1 may reside not only in exon 1 but also in its proximal intron 1.

The results shown are representative of three to five independent experiments.
The first GATA site within exon 1 is involved in GATA-1–mediated transcription. Because the results from the transient transfection experiments suggested that sequences from both exon 1 and intron 1 mediate transactivation by GATA-1, we evaluated the functional relevance of individual GATA sites to transactivation of the CCR3 gene. There are seven putative GATA sites in the Ex1-In1 sequence (Fig. 1C). GATA-1 binding occurs in two adjacent GATA sites within exon 1 (19), which correspond to the fourth and fifth GATA sites depicted in Fig. 1C. It also should be mentioned that the first exon initially identified by Zimmermann et al. (17) was found subsequently to be longer (16). The sequence identified by Zimmermann et al. (17) spanned +68 to +161 bp of exon 1 and thus lacked the first three GATA sites (Fig. 1C). To map the sequences responsible for functional GATA-1 binding, we created a series of point mutants in which the 5′-GATA-3′ of each GATA-containing sequence was replaced with 5′-TCGC-3′. K562 cells then were transfected with these constructs. Only the point mutant (mut1) in which the first GATA site was disrupted exhibited diminished transactivation activity. In agreement with that result, a mutant (del1) lacking the first GATA site exhibited similarly reduced activity. A decrease in transactivation was not observed in any of the other point mutants. A mutation in the nonconsensus GATC recognition sequence (mut6) also had no effect (Fig. 5A). These patterns of transactivation were not confined to K562 cells: similar results were obtained with A549 cells, which modestly express both CCR3 and GATA-1 (data not shown).

Inhibitory roles of the fourth, fifth, and eighth GATA sites in CCR3 transcription

Interestingly, mut4, mut5, and mut8 displayed significantly augmented transcription (compared with that of the wild-type Ex1-In1 construct), suggesting that the fourth, fifth, and eighth GATA sites inhibit transcriptional activation. To confirm their inhibitory roles, we created further point mutants, mut9 and mut10, which carried mutations in the fourth and fifth GATA sites in Ex1 (rather than in Ex1-In1), respectively. Both significantly increased transcription (Fig. 5B). Similarly, mutation of the eighth GATA site in In1 led to a profound increase in transcriptional activity. Moreover, mutations in these three GATA elements partially reversed the reduction in transcriptional activity resulting from mutation of the transcriptionally functional GATA site (mut1): mut1-4, mut1-5, and mut1-8 all displayed higher transcription than mut1 (Fig. 5C).

These data suggest that 1) the first GATA site is solely responsible for transactivation by GATA factors; 2) the fourth and fifth GATA sites, previously reported to be key elements mediating transactivation, in fact negatively modulate GATA-1 activity; and 3) a GATA site located in intron 1 also participates in the negative regulation of CCR3 gene transcription.

GATA-1 binding is not correlated with transactivation. Using EMSA, we examined whether the binding of GATA-1 to these GATA sites paralleled CCR3 transactivation. Nuclear extracts from...
K562 cells yielded the highest level of binding of GATA-1 to the first GATA site and a similar level of binding to the fourth GATA (Fig. 6A). The fifth and eight GATA sites exhibited intermediate levels of GATA-1 binding, and the second, third, and seventh GATA sites exhibited much less binding. As expected, GATA-1 did not bind to the sixth site, whose sequence was GATC instead of GATA. Thus, binding affinity did not correlate with the extent of activation. Binding complexes formed at the first (Fig. 6B), fourth, fifth, and eighth GATA sites (Supplemental Fig. 1) included GATA-1, as revealed by a supershift assay involving an anti-GATA-1 Ab. Abs raised to transcription factors, including GATA-2, PU.1, and AML-1α (whose encoding mRNAs were expressed in K562 cells, as analyzed by RT-PCR; data not shown), did not affect the binding of GATA-1 to the first (Fig. 6B) and other GATA elements (Supplemental Fig. 1). Our results suggest that the DNA-bound complex does not contain any of the aforementioned transcription factors (apart from GATA-1). Furthermore, the binding of GATA-1 to a hot probe containing the first GATA site was efficiently competed out by cold probes carrying the fourth and eighth GATA sites and less efficiently so by a cold probe carrying the fifth GATA site (Fig. 6C). Other combinations of hot and cold probes yielded similar patterns of inhibition (Supplemental Fig. 2), indicating that GATA-1 in isolation efficiently binds to any of these GATA elements. When nuclear extracts from different cell lines expressing varying amounts of endogenous GATA-1 were allowed to bind to a radiolabeled probe carrying the first GATA site, a DNA–protein complex was formed (Fig. 6D). No binding complexes were detected when a probe carrying the first GATA site was incubated with nuclear extracts from peripheral blood eosinophils (data not shown).

Treatment with BA induces CCR3 mRNA expression without enhancing GATA-1 binding and transactivation of the CCR3 gene. When K562 cells were treated with BA followed by IL-5, expression of CCR3 mRNA was induced, reaching a peak at day 5, and then declined to basal levels by day 7. Surface CCR3 expression was preceded by CCR3 gene transcription (Fig. 7A). A similar response was observed in HL-60 cells (data not shown). When the Ex1-In1 reporter plasmid was introduced to K562 cells that had been induced to differentiate through exposure to BA and IL-5 for 3 d, transactivation increased 3.6-fold compared with that of nontreated cells (Table I). However, this increase appeared to result from increased transfection efficiency in BA-treated cells, as demonstrated by a comparable increase (4.6-fold) in transactivation activity upon transfection with the pGL-Basic vector. Accordingly, levels of GATA-1 mRNA and protein were not increased by BA treatment (data not shown). To evaluate whether the increase in transactivation activity was caused by increased GATA-1 binding in vivo, we performed ChIP assays using ge-
nomic DNA isolated from undifferentiated cells and differentiated K562 cells. In untreated cells (as in A549 cells), GATA-1 weakly occupied the GATA elements in exon 1 and intron 1. BA treatment had little effect on GATA-1 binding (Fig. 7B), suggesting that butyric treatment does not stimulate the binding of GATA-1 to the GATA elements. Moreover, we examined whether there would be differential binding of GATA-1 to the positively and negatively acting GATA elements within exon 1 in uninduced and maximally induced CCR3 mRNA states. ChIP assays were performed with the two additional primer sets shown in Fig. 7C and K562 cells treated with or without BA and IL-5. We found that both positively and negatively acting GATA sites were bound to GATA-1 regardless of CCR3 expression levels, lowering the likelihood of differential binding of GATA-1 to these GATA sites in this cell type. Instead, acetylation of histone 3 at K9 increased in the studied regions of the CCR3 gene, whereas methylation of histone 3 at K9 remained unchanged (Fig. 7D), suggesting that BA influences chromatin remodeling.

Discussion
We mapped a GATA site necessary for GATA-1–mediated transactivation to exon 1 of the CCR3 gene. ChIP analysis initially...
enables us to narrow our search for GATA-1 binding sites to sequences in exon 1 and its proximal intron 1 rather than sequences upstream of exon 1 (Fig. 3). Subsequently, transient transfection experiments involving reporter genes linked to sequences containing exon 1, intron 1, or both showed that both exon 1 and intron 1 contain sequences that regulate GATA-1–mediated transactivation. Moreover, transactivation was essentially proportional to levels of endogenous GATA-1 (Fig. 4).

Table I. Transactivation of CCR3 reporter plasmids in K562 cells treated and untreated with BA

<table>
<thead>
<tr>
<th></th>
<th>pGL3-Basic</th>
<th>Ex1-In1</th>
<th>Fold Increase (Ex1-In1/pGL3-Basic)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BA</td>
<td>4.0 ± 0.3</td>
<td>177.9 ± 7.4</td>
<td>44.3 ± 1.8</td>
</tr>
<tr>
<td>+BA</td>
<td>18.4 ± 0.2</td>
<td>641.5 ± 7.1</td>
<td>34.8 ± 0.4</td>
</tr>
</tbody>
</table>

Enabled vector (pGL3-Basic) and the Ex1-In1 reporter construct were introduced by transfection to K562 cells that had been treated with BA for 1 d and cultured with IL-5 for an additional 2 d. Values represent luciferase activities.

To compare reporter transactivation in untreated and treated K562 cells, the luciferase activity for Ex1-In1 construct was divided by that for the empty vector. Transfections were performed in triplicate. Data represent the mean ± SEM of two independent experiments.

The third important observation in the current study was that the level of transactivation of the CCR3 gene by GATA-1 may depend on the net effect of its interaction with GATA elements that act as both enhancers and repressors of transactivation. Indeed, the fourth GATA site binds to GATA-1 with high affinity (comparable to that of the first GATA site; Fig. 6A) without increasing transactivation (Fig. 5A, 5B). Similarly, the fifth and eighth GATA sites, which displayed intermediate levels of GATA-1 binding, made no positive contribution to transactivation. Instead, point mutations of these three sites resulted in a significant increase in transcriptional activity (Fig. 5A, 5B), indicating that these GATA sites may act as negative cis elements controlling transactivation of the CCR3 gene by GATA-1. We then hypothesized that the reduction in transcriptional activity resulting from mutation of the first GATA site might be, at least in part, reversed by mutation of the GATA sites that negatively regulate transcription. Indeed, this was the case (Fig. 5C).

This finding is in agreement with a previous report (33), which demonstrated that GATA-1’s two zinc fingers interact to influence DNA binding and transactivation: the N-terminal finger (and adjacent linker region) alters the binding specificity of the C-finger and even inhibits the ability of the C-finger to recognize consensus GATA sequences. Moreover, because high-affinity binding does not always lead to transactivation, some GATA sites may have other roles. Indeed, the fourth, fifth, and eighth GATA sites in the Ex1-In1 sequence of the CCR3 gene may act to inhibit transactivation. We therefore reason that transactivation of the CCR3 gene by GATA-1 may be influenced by the nature of the two GATA sites that the two zinc fingers of GATA-1 engage during binding. Even if these GATA sites are not located in tandem in the regulatory region, they may be brought into proximity through looping, because it was shown that DNA bending may be induced by the binding of GATA-1 (41) and possibly other transcription factors. In this sense, the more favorable scenario for transactivation would involve simultaneous binding of GATA-1 to the first and second or sixth GATA sites and not the fourth, fifth, or eighth GATA sites, mutations in which increased transactivation. These three GATA sites may act as sinks for GATA-1, reducing the amount of protein available for binding to the positive regulatory elements. Furthermore, although eosinophil-specific genes commonly have GATA elements in their regulatory regions, GATA-1 binding may affect GATA-containing promoters or enhancers in individual eosinophil-specific genes differently, because these genes differ in the number and spacing of GATA sites in their regulatory regions. The number and spacing of these GATA elements may collectively influence transactivation, in a sequence-specific manner, by altering the conformation of GATA-1.

Our EMSA and point mutation analyses allowed us to determine the GATA-1 binding affinities and roles of individual GATA elements in exon 1 and intron 1 of the CCR3 gene. However, it remains to be shown whether these individual GATA elements have similar GATA-1 binding affinities and roles in the context of an intact CCR3 gene with a particular chromatin structure and pattern of DNA methylation. Examination of the exon 1 and intron 1 sequences reveals that the fourth and fifth GATA elements immediately follow CpG dinucleotide sequences (fourth, 5′-tacctc′-3′, and fifth, 5′-tacctc′-3′, where underlined and bold text denotes the GATA elements and CpG sites, respectively). A recent study showed that CpG-poor promoters are hypermethylated in somatic cells, which does not preclude their activity (42). Given that DNA methylation interferes with the binding of transcription factors (43), methylation of these GATA sites would be expected to greatly influence binding affinity for GATA-1 and to have functional consequences.

Although GATA-1 acts as a central regulator of CCR3 gene transcription, it is not likely to be sufficient for the induction of CCR3 mRNA expression. First, K562 cells do not constitutively express CCR3 mRNA despite abundant expression of GATA-1 (Fig. 2). Second, BA induces CCR3 mRNA without increasing cellular GATA-1 levels, binding of GATA-1 to sequences in the CCR3 regulatory regions (Fig. 7), and reporter gene transactivation (Table I). Moreover, transcription factors such as C/EBP...
and PU.1 synergize with GATA-1 to upregulate MBP transcription in developing eosinophil progenitors (39), whereas GATA-2 activates the EDN promoter in developing eosinophil progenitors (28). The cis-acting elements for these transcription factors are clustered with elements for AML-1a/RUNX1 in the regulatory regions of the CCR3 gene. Nevertheless, they do not seem to account for the inducibility of CCR3 mRNA expression, because K562 cells constitutively express all of these factors (data not shown). Therefore, interplay among GATA factors, PU.1, C/EBP, and AML-1a/RUNX1 is not sufficient per se to induce CCR3 transcription.

Treatment with BA, followed by IL-5, led to the induction of CCR3 mRNA expression in K562 cells (Fig. 7A) and HL-60 cells (data not shown). A marked parallel increase in histone 3 K9 acetylation was observed (Fig. 7D), suggesting that the induction of CCR3 mRNA requires changes in chromatin structure. It has been reported that acetylation of K9 is critical for the recruitment of TFIIID (44). In addition to chromatin remodeling, BA and/or IL-5 treatment might activate signals that are necessary for CCR3 mRNA induction. Activation of p38 MAPK is known to be involved in BA-mediated differentiation of K562 cells to erythroid cells (45) and also occurs during IL-5-induced differentiation of bone marrow-derived differentiated eosinophils (46). Given the intimate relationship between the differentiation of K562 cells and eosinophils and CCR3 expression, MAPK signaling, which is unlikely to be activated by the mere presence of GATA-1 and other transcription factors that influence the expression of eosinophil-specific genes, may be necessary for the expression of CCR3 mRNA to be induced.

In summary, we have reassigned the GATA site that is essential for transactivation of the CCR3 gene. However, this GATA site does not alone account for GATA-1-mediated regulation but instead exerts its effects in conjunction with other positively and negatively acting GATA sites. An integrated transactivation signal thus is generated through the dynamic interaction of GATA-1 with these various GATA sites in the regulatory region of the CCR3 gene.

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Disclosures

The authors have no financial conflicts of interest.

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