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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 Kyung 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 eosinophilic genes 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 bound to virtually all seven putative GATA elements present in exon 1–intron 1, the first GATA site in exon 1 exhibited the highest 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: 6866–6875.

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...
(EDN), contain functional GATA elements at which GATA-1 interacts with other transcription factors to activate transcription (26–28). Zimmermann et al. (19) showed that GATA-1 binds to a double-GATA element within exon 1 of the CCR3 gene. However, the functional relevance and precise role of these GATA elements in the transcriptional regulation of the CCR3 gene remain to be determined.

GATA-1, one of six members of the GATA family, binds to consensus (A/T)GATA(A/G) sequences through a DNA-binding domain comprising two characteristic zinc finger motifs (29–31). The C-terminal finger (C-finger) is essential for GATA-1 function, because it is both necessary and sufficient for high-affinity binding to consensus sequences (32). The N-terminal finger, which does not bind DNA independently, influences C-finger binding. It may stabilize or disrupt binding or alter the binding specificity of the C-finger (33). More importantly, even high-affinity binding of GATA-1 does not necessarily lead to transactivation (33). In general, GATA-1 does not bind to its DNA recognition sequences with equal affinity, even those that include an identical consensus GATA binding sequence. The basis for this differential binding affinity might lie in differences in the sequences flanking the consensus GATA binding sequence and the orientation and spacing of the GATA elements to which GATA-1’s two zinc fingers bind.

Despite the fact that numerous studies have focused on CCR3 gene expression, CCR3 ligand specificity, the development of CCR3 antagonists, and the role of CCR3 in eosinophil trafficking in vitro and in vivo, there is a surprising paucity of information on CCR3 gene transcription at the molecular level. In the current study, we explored the molecular basis of CCR3 gene expression and focused on GATA-1’s specific mode of action. By analyzing constructs containing a few dozen point mutants and a deletion mutant of CCR3 regulatory sequences with chromatin immunoprecipitation (ChIP) and EMSA assays, we identified a functional GATA element. This GATA element, which was not recognized previously as being important, does not appear to meet the criteria that define the proposed double-GATA element. Moreover, additional GATA elements within the regulatory regions of the CCR3 gene may participate in the fine-tuning of CCR3 gene transcription.

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 embryo 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 × 10^6 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 40–60% 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 SCHB-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 TRIZ reagent (Molecular Research Center, Cincinnati, OH). First-strand cDNA was synthesized from 1 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′-ATGCGATGTGAACGCTCCTC-TCAG-3′ and CCR3 reverse primer 5′-TTGTCCTGGCTACAGTCATTTC-CC-3′; GATA-1 forward primer 5′-GCCCTGACTTTCAGACTCC-3′ and GATA-1 reverse primer 5′-CGAGCTCTGAATCCTACCTCC-3′; GAPDH forward primer 5′-CTGCTTCAACCATGGAGA-3′ and GAPDH reverse primer 5′-CGGCCATCACGGCCACGT-3′.

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 FACS-Calibur 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% NaN3, 0.5% 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 microliters 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 (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, transfection, 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′-TGTCG-3′. Amplification was performed using four primers (including two overlapping primers for each mutation) and Pyrobest Q (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 III. Cell lines were transfected with the reporter plasmids using Lipofectamine 2000 reagent (Invitrogen Life Technologies). pSV-β-galac-
MAPPING A FUNCTIONAL GATA-1 BINDING SITE IN THE CCR3 GENE

GATA-1 binding to sequences in exon 1 and proximal intron 1 of the CCR3 gene

It was demonstrated previously that the key regulatory sequences for CCR3 gene transcription reside not only in exon 1 (17, 18) but also in proximal intron 1 (16). A subsequent study identified a palindromic double-GATA site in exon 1 of the CCR3 gene as a GATA-1 binding site, as judged by EMSA, but did not analyze its functional effects (19). To investigate the involvement of these regions of the CCR3 gene in GATA-1 binding, four different regions were selected for ChIP analysis (Fig. 1A): a promoter sequence (−280 to −98 bp); exon 1 (+1 to +161 bp); proximal intron 1 (+162 to +405 bp); and a protein-coding sequence located −22 kbp downstream of exon 1. To avoid confusion, it should be mentioned that the numbering was according to the longest exon 1 (161 bp in length), as described previously (16). Thus, the length of the exon 1 is 67 bp longer than that of exon 1 identified by Zimmermann and his colleagues (17) (the first nucleotide of the exon 1 that those authors described corresponds to the 68th nucleotide of the exon 1 that we defined in this study; Fig. 1C). The ChIP assay was performed on cross-linked genomic DNA from A549 cells. An anti–GATA-1 Ab was able to detect exon 1- and intron 1-bound GATA-1 but failed to pull down GATA-1–bound promoter and the protein-coding CCR3 DNA sequences (Fig. 3). No signal was detected with control Ab. An anti-histone 3 Ab efficiently pulled down histone-DNA complexes in the same region, allowing us to compare ChIP efficiency and/or specificity. These results suggest that GATA-1 binds to sequences in exon 1 and proximal intron 1 of the CCR3 gene in cells that modestly and constitutively express GATA-1 and CCR3. Thus, the intron 1 sequence also may contribute to transactivation of the CCR3 gene by GATA-1. In addition, the failure of GATA-1 to bind to promoter sequences upstream of exon 1 suggests a minimal role for these sequences in CCR3 transactivation (16, 17).

Both exon 1 and intron 1 contain sequences that are important for transactivation by GATA-1. On the basis of our ChIP data and the findings of previous studies (16, 18, 19), we investigated the contribution of exon 1 and the intron 1 sequences to transcription activation of the CCR3 gene. To this end, we generated reporter plasmids bearing sequences from exon 1 (named Ex1), its 3′ proximal intron 1 sequence (In1), or both exon 1 and intron 1 (Ex1-In1) (Fig. 1B). Four different cell lines were transfected with these three constructs, and luciferase activities were measured. The three reporters produced almost identical patterns of transactivation in all four cell lines studied. The Ex1-In1 construct yielded the highest transactivation in all cells tested, whereas Ex1 and even In1 retained substantial transactivation, especially in 293T and K562 cells, which expressed higher levels of GATA-1 than A549 and NC-IH292 cells. Levels of reporter transactivation were essentially proportional to the endogenous levels of GATA-1 (Fig. 4A). In support of the results, the transactivation of the three constructs was reduced by half by DN-GATA-1 (Fig. 4B). The inhibitory response by DN-GATA-1 was dose-dependent, even in the case of the In1 construct (Fig. 4C). Furthermore, introduction of GATA-1 siRNA led to a decrease in transcription activity of
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.

**FIGURE 2.** Correlation between the levels of expression of CCR3 and GATA-1 in various cell types. A and B, Expression of CCR3 and GATA-1 mRNA and protein was analyzed in cell lines and peripheral blood eosinophils. Protein expression was analyzed by FACS (CCR3) and Western blotting (GATA-1), and mRNA expression was analyzed by RT-PCR. C, CCR3 and GATA-1 expression in developing eosinophils. Unfractionated umbilical cord blood mononuclear cells were cultured as described previously (34). Expression of GATA-1 and GAPDH mRNA was analyzed by multiplex RT-PCR. GAPDH mRNA and protein were used as loading controls. The results shown are representative of three to five independent experiments.
and mut1–8 all displayed higher transcription than mut1 (Fig. 5C)
transcriptionally functional GATA site (mut1): mut1–4, mut1–5,
duction in transcriptional activity resulting from mutation of the
mutations in these three GATA elements partially reversed the re-
a profound increase in transcriptional activity. Moreover, muta-
(Fig. 5B).
Ex1-In1), respectively. Both significantly increased transcription
mutations in the fourth and fifth GATA sites in Ex1 (rather than in
we created further point mutants, mut9 and mut10, which carried
inhibit transcriptional activation. To confirm their inhibitory roles,
construct), suggesting that the fourth, fifth, and eighth GATA sites
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 transactivational 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 con-
fined 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 aug-
mented 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, muta-
tions in these three GATA elements partially reversed the re-
duction 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 trans-
activation, 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

FIGURE 3. GATA-1 binds to regulatory sequences in exon 1 and prox-
imal intron 1 of the CCR3 gene. A549 cells were fixed in formaldehyde and
sonicated. A rat anti-human GATA-1 Ab (N6; Santa Cruz Biotechnology)
was used to pull down DNA fragments bound to GATA-1. An anti-histone 3
Ab and a rat IgG were used as positive and negative controls, respectively.
D.W indicates no DNA template. The pulled-down DNA fragments were
amplified by conventional PCR using the primers listed in Supplemental
Table I. Different fragments were named as shown in Fig. 1A: Fragment 1,
the proximal promoter; Fragment 2, exon 1; Fragment 3, proximal intron 1;
Fragment 4, the protein-coding region of the CCR3 gene. The results shown
are representative of three independent experiments.

FIGURE 4. Transactivation of CCR3 is related to levels of endogenous
GATA-1 and is inhibited by DN-GATA-1 and GATA-1 siRNA. A, Four
different cell lines were transfected with Ex1-In1, Ex1, and In1 reporter
constructs, whose transcription activities then were measured. B, K562
cells were cotransfected with DN-GATA-1 and each of the three con-
structs. C, The Ex1-In1 and In1 constructs were introduced to K562 cells
together with increasing concentrations (0.01, 0.05, and 0.1 μg) of DN-
GATA-1. Data represent the mean ± SEM of at least three independent
experiments. D, K562 cells were cotransfected with the Ex1-In1 construct
and GATA-1 siRNA or scrambled siRNA (1, 5, 10, and 25 nM). Results are
the average of two independent experiments that are set in triplicate. The
aliquot of the transfected cells was tested for GATA-1 protein levels by
Western blot analysis.

FIGURE 2. Fragments of the CCR3 gene. These fragments represent
three independent enzymes. A, Exon 1 and intron 1 map the sequences
responsible for functional GATA-1 binding, whereas the proximal promoter
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 ad-
Junctional 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 transactivational 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 con-
fined to K562 cells: similar results were obtained with A549 cells,
which modestly express both CCR3 and GATA-1 (data not shown).
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 eighth 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). 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). 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).

When the 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-
omic 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

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**FIGURE 6.** Binding of GATA-1 to the putative GATA sites. A, Eight different radioactive probes (indicated in Fig. 1C) were incubated with nuclear extracts from K562 cells. Competitors were used to test the specificity of binding: w and m represent wild-type and mutant sequence-harboring cold probes, respectively. The sequence 5'-GATA-3' in the w probes was replaced with 5'-TCGC-3' in the m probes, except for probe 6, in which the 5'-GATC-3' was replaced with 5'-AGCT-3'. B, Nuclear extracts from K562 cells were incubated with Abs raised against different transcription factors, followed by 32P-labeled probe 1. S and C indicate specific and control Abs, respectively. C, Probe 1 was incubated with varying concentrations (10 and 50 molar excess) of unlabeled probes containing the first, fourth, fifth, and eighth GATA sites. D, Probe 1 was incubated with nuclear extracts from the cell lines tested in the transfection experiments. Arrows and the arrowhead indicate specific binding and a supershift, respectively.

**FIGURE 7.** Expression of CCR3, GATA-1 binding, and the modification of histone 3 at K9 in the regulatory regions of the CCR3 gene during BA-induced differentiation of K562 cells. A, K562 cells were cultured with 0.5 mM BA for 1 d and then with IL-5 for up to 7 d. CCR3 mRNA levels and cell surface expression of CCR3 protein were measured. The results shown are representative of three experiments. B–D, K562 cells were cultured with BA for 1 d and then with IL-5 for an additional 2 d. The cultures were subjected to ChIP analysis using Abs raised against GATA-1 (B, C) and modified forms of histone 3 (D). In B, different fragments were named as shown in Fig. 1A. In C, two additional primer sets were used for the ChIP assay, and the open box and ovals represent exon 1 and GATA sites within exon 1, respectively. In D, the results shown are representative of three independent experiments (the results of the other two experiments revealed similar patterns of histone modification).
In addition, although the MBP P2 promoter contains a double-GATA sequence, these GATA sites significantly suppress transcriptional activity. Mutations in 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). More conclusively, only two mutants, one in which the first GATA site was disrupted and a second in which it was missing, exhibited severely impaired transcription (Fig. 5). In addition, the first GATA site exhibited high-affinity binding for GATA-1 (Fig. 6). This functional GATA element is different from the one that was proposed previously to be critical for GATA-1–mediated transactivation of the CCR3 gene (19); the authors of a previous report claimed, based on the results of EMSA analysis, that the fourth and fifth GATA sites constituted a canonical double-GATA site responsible for the regulation of CCR3 expression by GATA-1. Moreover, the functional GATA site does not meet the criteria that define the proposed double-GATA element, which is postulated to be a key sequence for the regulation of eosinophil-specific genes by GATA-1 (3). Such double-GATA sites have been identified in several genes. Notably, a double-GATA sequence in the mouse GATA1 gene is essential for the activation of its promoter. Furthermore, ablation of this palindromic GATA1 sequence results in loss of the eosinophil lineage (20). Similar double-GATA sequences are present in the promoter or enhancer regions of the eosinophil-specific genes encoding CCR3, MBP, and human IL-5Rα (3). However, it is not clear whether the double-GATA site is essential for the transcriptional activation of eosinophil-specific genes. Charcot-Leyden crystal protein (27) and gp91phox (40) are encoded by eosinophil-specific genes. Charcot-Leyden crystal protein (27) and gp91phox (40) are encoded by eosinophil-specific genes that are expressed in eosinophils and neutrophils, respectively. Charcot-Leyden crystal protein (27) and gp91phox (40) are encoded by eosinophil-specific genes that are expressed in eosinophils and neutrophils, respectively.

 enabled 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). More conclusively, only two mutants, one in which the first GATA site was disrupted and a second in which it was missing, exhibited severely impaired transcription (Fig. 5). In addition, the first GATA site exhibited high-affinity binding for GATA-1 (Fig. 6). This functional GATA element is different from the one that was proposed previously to be critical for GATA-1–mediated transactivation of the CCR3 gene (19); the authors of a previous report claimed, based on the results of EMSA analysis, that the fourth and fifth GATA sites constituted a canonical double-GATA site responsible for the regulation of CCR3 expression by GATA-1. Moreover, the functional GATA site does not meet the criteria that define the proposed double-GATA element, which is postulated to be a key sequence for the regulation of eosinophil-specific genes by GATA-1 (3). Such double-GATA sites have been identified in several genes. Notably, a double-GATA sequence in the mouse GATA1 gene is essential for the activation of its promoter. Furthermore, ablation of this palindromic GATA1 sequence results in loss of the eosinophil lineage (20). Similar double-GATA sequences are present in the promoter or enhancer regions of the eosinophil-specific genes encoding CCR3, MBP, and human IL-5Rα (3). However, it is not clear whether the double-GATA site is essential for the transcriptional activation of eosinophil-specific genes. Charcot-Leyden crystal protein (27) and gp91phox (40) are encoded by eosinophil-specific genes that contain a single-GATA site in their promoters, and mutation of these GATA sites significantly suppresses transcriptional activity. In addition, although the MBP P2 promoter contains a double-GATA site, mutation of one of the two GATA subsites is sufficient to inhibit transcriptional activity (26). Elsewhere, the two GATA elements in the EDN promoter are ~600 bp apart. A mutation in either site sharply reduces promoter activity (28). Although these studies do not completely rule out the possible requirement for a double-GATA site for transactivation, neither do they support the double-GATA hypothesis.

A second 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). Consequently, the level of transcriptional activation does not necessarily correlate with DNA binding affinity. 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 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.

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>pGL3-Basic</th>
<th>Ex1-In1</th>
<th>Fold Increase</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>

*Empty 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-3 for an additional 24 h. 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.

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

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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-1/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-1/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 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 reassembled 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.

Acknowledgments

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References


