Brief report

Deletion of the major GATA1 enhancer HS 1 does not affect eosinophil GATA1 expression and eosinophil differentiation

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Expression of the myeloid transcription factor GATA1 is required for early stages of eosinophil differentiation. Defining mechanisms regulating eosinophil GATA1 expression will be important to understand development of this lineage. However, the *cis*-elements required for eosinophil GATA1 expression are not fully characterized. Previous work identified HS 1 as a major GATA1 enhancer, but its role in eosinophil GATA1 expression is unclear. Here, we show that mouse HS 1 deletion leaves eosinophil GATA1 mRNA expression and eosinophil differentiation unaffected. Chromatin isolated from eosinophils and encompassing HS 1 is weakly enriched for acetylated histones H3/H4. HS 1 deletion does not alter eosinophil GATA1 locus histone acetylation. In eosinophils, GATA1 and CCAAT/enhancer binding protein ϵ (C/EBPϵ) do not bind HS 1 but bind selectively a *cis*-element in the first GATA1 intron. Thus, HS 1 is not required for eosinophil GATA1 expression. Instead, this study suggests a previously unsuspected role for the GATA1 intron element for this function. (Blood. 2004;104:89-91)

Introduction

The transcription factor GATA1 plays a central role in myelopoiesis. It is expressed in the common myeloid progenitor (CMP) and expression is selectively maintained in red cells, megakaryocytes, eosinophils, and mast cells but extinguished in granulocytes/macrophages.[1] It promotes differentiation of myeloid progenitors to red cells, megakaryocytes, and eosinophils at the expense of granulocytes/macrophages.[1-3] It is required for terminal maturation of red cells, megakaryocytes, and mast cells and at the earliest stages of eosinophil differentiation (reviewed in Cantor and Orkin[4] and McNagny and Graf[5]). These observations argue that defining the molecular mechanisms regulating GATA1 expression will be one important component in dissecting the molecular control of myeloid differentiation.

In mice, 3 *cis*-acting sequences (*cis*-elements)—the hematopoietic exon 1 erythroid (IE) promoter (this includes both promoter proximal and distal elements within approximately 900 base pairs from the transcriptional start sites in the exon associated with this promoter), an upstream enhancer HS 1/G1HE (hereafter referred to as HS 1), and an element in the first GATA1 intron (HS 4/5)—direct reporter transgene expression to definitive red cells and megakaryocytes.[6,7] The intron element is not required for primitive red cell GATA1 expression.[7] Germ line HS 1 deletion almost completely abolishes megakaryocyte GATA1 expression but leaves red cell GATA1 expression unaffected,[8,9] suggesting that additional sequences compensate for HS 1 absence in red cells but not megakaryocytes.

By contrast, little is known about sequences controlling eosinophil GATA1 expression. Although sequences in the IE promoter are important,[10] there are conflicting data on the function of HS 1. In 2 reports, all known GATA1 *cis*-elements (including HS 1) failed to target transgene expression to eosinophils.[6,11] In contrast, another study suggests that a similar combination of GATA1 regulatory sequences can do so.[12] One potential reason for these conflicting results is that transgene expression can be modulated by position of integration. Therefore, to directly test the nonredundant function of HS 1 in eosinophil GATA1 expression, we asked whether eosinophil GATA1 expression and eosinophil development are perturbed in mice deleted for HS 1.

Study design

Mice and cell culture

ΔneoΔHS[8] and wild-type littermate female mice were mated with interleukin 5 (IL-5) homozygous transgenic male mice.[13] Male offspring were genotyped for presence or absence of the HS 1 enhancer by using primers 5′-AATCAGGAATGCAACATCTC-3′ and 5′-ACTCTTGCTCTCTTTTGCAG-3′ and for presence of the IL-5 transgene by using previously published primers.[10] A Pentra 60 (ABX Diagnostics, Montpellier, France) automated counter was used to determine blood counts. Blood smears were stained with May-Grünwald-Giemsa (MGG) stain (Sigma, Poole, United Kingdom). Megakaryocytic cell line L8057 was cultured as previously described.[14]

Eosinophil purification

Peripheral blood red cells were pelleted by mixing 1 volume of blood with 5 volumes of 1.25% dextran. White blood cells were pooled with splenocytes,

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and the remaining red cells were lysed by hypotonic shock. Cells were labeled with anti-CD2 (reference 553109), anti-B220 (reference 553084), and anti-Gr1 (reference 553123) (Pharmingen, Oxford, United Kingdom) and removed using magnetic beads coated with goat antirat immunoglobulin G (IgG) and a lactic dehydrogenase (LD) depletion column (Miltenyi Biotech, Bergisch Galdbach, Germany). Unlabeled cell population was routinely more than 95% eosinophils (assessed by MGG staining). Fast Green and Neutral Red stains (Sigma) were performed by using standard protocols. Micrograph images were taken with a BX60 microscope and a × 40/0.75 objective (Olympus, London, United Kingdom), using a Qicam camera (Qimaging, Burnaby, Canada) and Openlab software (Improvision, Coventry, United Kingdom).

GATA1 mRNA quantitation

cDNA, produced from total RNA by standard protocols, was used in real-time polymerase chain reaction (PCR) according to recommended ABI protocols on a ABI7000 Thermocycler (Applied Biosystems, Warrington, United Kingdom). Primers and probe used included 5′-AGAGAAGCTGAG-GCCTACAGA-3′, 5′-CAGGAATTCCCTCCATACTGTTGAG-3′, and 5′-FAM-CACTCCCCAGTCTTTC-3′-NFQ (Applied Biosystems). GATA1 signal was normalized to both β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), by using primers and probes from Eurogentec (Romsey, United Kingdom) (RT-CKYD-ACTB and RT-CKYD-GAPD).

Chromatin immunoprecipitation

From 1 to 3×10^7 cells were used for immunoprecipitation by using conditions suggested by manufacturer's protocol (Upstate Biotechnology, Lake Placid, NY), except for GATA1.[15] Polyclonal antibodies used included anti-acetylated H3 (no. 06-599; Upstate Biotechnology), GATA1, CCAAT/enhancer binding protein ϵ (C/EBPϵ) (no. sc-265 and no. sc-158; Santa Cruz, Calne, United Kingdom). For each cell type and antibody, at least 3 independent chromatin preparations were used for immunoprecipitation. Details of the chromatin immunoprecipitation (ChiP) assays, real-time PCR experiments, and sequences of the primers and probes used in Taqman PCR are set out in the supplemental table at the *Blood* website (see the Supplemental Table link at the top of the online article).

Results and discussion

Blood counts from mice deleted for HS 1 (ΔneoΔHS),[8] and normal littermate controls were analyzed (Table 1). As the normal eosinophil count is low, a subtle eosinophil phenotype may be missed. To increase the eosinophil count, ΔneoΔHS were bred to transgenic mice overexpressing IL-5.[13] Thus, ΔneoΔHS mice heterozygous for the IL-5 transgene and IL-5 heterozygous transgenic littermate controls were studied. ΔneoΔHS mice and ΔneoΔHS/IL-5 transgenic mice have eosinophil counts comparable to control wild-type (WT) and IL-5 transgenic mice (WT/IL-5 Tg), respectively.

Table 1. Peripheral blood hematology

	WT	ΔneoΔHS	WT/IL-5 Tg	ΔneoΔHS/ IL-5 Tg
Hemoglobin, (g/dL)	14.9 (0.8)	13.3 (3.1)	16.4 (1.5)	16.4 (1.5)
Platelet count, 10^3/mm	868.0 (122)	121.0 (31.0)	798.0 (99.0)	95.0 (14.0)
White cell count, 10^3/mm	5.6 (1.3)	4.6 (0.6)	34.7 (8.9)	34.6 (14.0)
Neutrophils, (%)	24.8 (3.0)	38.0 (5.3)	12.3 (3.5)	12.0 (1.5)
Lymphocytes, (%)	73.3 (2.9)	60.0 (4.5)	46.8 (8.6)	45.6 (3.5)
Eosinophils, (%)	2.2 (0.6)	2.0 (0.5)	40.8 (10.6)	42.4 (4.3)

Peripheral blood hematology was determined in male wild-type mice (WT), male mice deleted for HS 1 (ΔneoΔHS), male IL-5 heterozygous transgenic mice (WT/IL-5 Tg), and compound heterozygous IL-5/ΔneoΔHS male mice. Four mice were tested in each group, and average values are shown. Hemoglobin, platelet count, and white cell count values were obtained from an automated counter. Manual white cell differential counts from blood smears stained with MGG stain show percentage of neutrophils, lymphocytes, and eosinophils from 400 cells counted.

Values in parentheses indicate ± 1 SD.

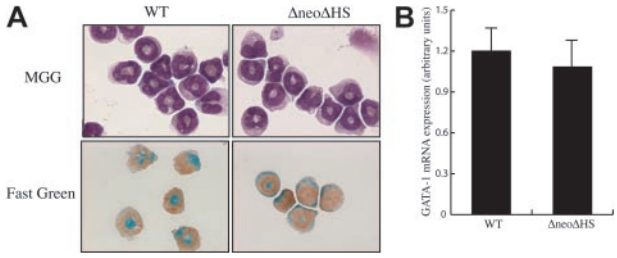

Figure 1. HS 1 is not required for eosinophil GATA1 expression and eosinophil maturation. (A) Morphology of eosinophils purified from blood and spleen of IL-5 transgenic mice with an intact (WT) or deleted HS1 enhancer (ΔneoΔHS). After cytospin, cells were stained with MGG stain to check their morphology or with Fast Green and Neutral Red to document presence of cytoplasmic green eosinophilic granules. Original magnification, × 400. (B) GATA1 mRNA levels were determined by Real-Time Taqman PCR in purified blood eosinophils from IL-5 transgenic mice with an intact (WT) or deleted HS1 (ΔneoΔHS) enhancer. The results are from 4 mice in each group. Error bars represent ± 1 SD.

Morphologic analysis by MGG and Fast Green stains shows comparable eosinophil maturation in wild-type and ΔneoΔHS mice (Figure 1A). Consistent with these observations, GATA1 mRNA expression in purified eosinophils with intact and deleted HS 1 is comparable when quantitated by Real-Time Taqman PCR (Figure 1B). For the first time, these observations demonstrate that both eosinophil GATA1 mRNA expression and eosinophil differentiation are unaffected in absence of HS 1.

Chromatin enriched for acetylated core histones H3/H4 is associated with active *cis*-elements (reviewed in Bulger et al[16] and Johnson and Bresnick[17]). Furthermore, *cis*-element deletion can perturb long-range histone acetylation.[18] Therefore, we quantitated relative H3/H4 acetylation at 11 points in the mouse GATA1 locus from coordinates −8.1 to +22.4 (in kilobases with respect to the transcriptional start of the *GATA1* gene in the IE promoter) in purified primary eosinophils with and without HS 1. As H3 and H4 acetylation profiles were similar, only H3 results are presented (Figure 2A). Chromatin isolated from eosinophils with intact HS 1

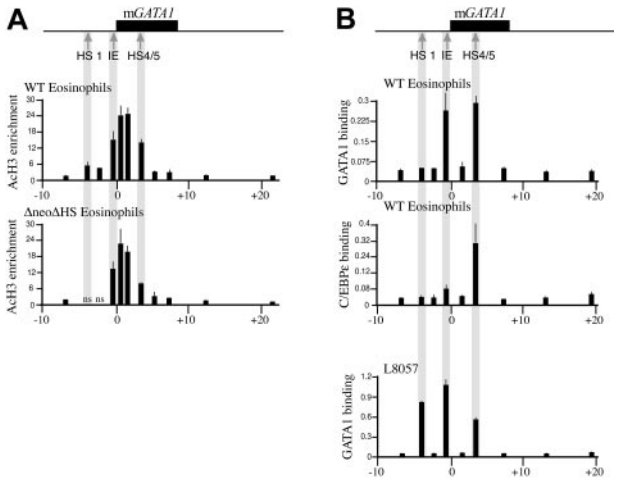

Figure 2. Histone acetylation profile and transcription factor binding in the m*Gata1* locus. (A) The mGATA1 locus is shown at the top. The *Gata1* gene is depicted as a black box, and the position of *cis*-elements HS 1, IE promoter, and the intron element (HS4/5) are marked by gray arrows. Below, at different points along the GATA1 locus (x-axis, the coordinates are in kilobases), the degree of enrichment of acetylated histone H3 (black rectangles with 1 SD error bars indicated, y-axis) in eosinophils with intact HS 1 (WT) or deleted for HS 1 (ΔneoΔHS) is shown. Acetylation at HS 1, IE promoter, and HS 4/5 is highlighted by gray stripes. Ns indicates no signal. (B) Above, the m*Gata1* locus is shown as in panel A. Below, the relative degree of binding (black rectangles with 1 SD error bars indicated, y-axis) of transcription factors GATA1 and C/EBPϵ in wild-type eosinophils and GATA1 in the megakaryocytic cell line, L8057, is shown at different positions along the m*Gata1* locus (x-axis).

is enriched approximately 7- to 12-fold for acetylated H3 from the IE promoter through to the regulatory element in the first intron compared with neighboring points in the locus. Contrastingly, chromatin containing HS 1 is enriched only 2- to 3-fold for acetylated H3. The same pattern of acetylation is seen in absence of HS 1. The pattern of acetylation with isotype control antibodies revealed no increased acetylation at regulatory elements compared with neighboring points in the locus (data not shown). These results suggest that histone acetyltransferase activity is mainly recruited to the IE promoter and intron element and show that HS 1 deletion does not perturb long-range GATA1 locus chromatin structure. In contrast, in the megakaryocytic cell line L8057 chromatin associated with HS 1 was enriched in acetylated H3 and H4 (B.G., unpublished observation, December 2003). It is noteworthy that HS 1 is required for megakaryocyte-GATA1 expression.[8]

Finally, we determined in vivo binding of transcriptions factors that potentially regulate GATA1 expression in primary purified eosinophils (Figure 2B). We studied GATA1 because it is likely to autoregulate its own expression[19,20] and because critical GATA binding sites have been demonstrated in HS 1 and the IE promoter.[19,21] We also assayed C/EBPϵ and C/EBPα binding in the GATA1 locus as mice null for expression of these genes have impaired eosinophil differentiation.[22,23] As a positive control for GATA1 binding at HS 1, we tested the megakaryocytic cell line, L8057 (Figure 2B). In wild-type eosinophils, GATA1 binding was detected at the IE promoter and intron element but not at HS 1. Interestingly, C/EBPϵ was also detected at the intron element with weak binding at the IE promoter. Consistent with this finding there are 2 C/EBP sites in the IE promoter and one site in HS4/5 within 600 base pairs either side of where the primer pairs are located in the analysis, suggesting that these C/EBP motifs do function in vivo. In contrast, C/EBPα binding was not present in the GATA1 locus. In L8057 cells, strong binding of GATA1 was detected at HS 1 in addition to the IE promoter and the intron element. Importantly, we did not see any binding with isotype control antibodies at any location in the GATA1 locus (data not shown).

For the first time, these data demonstrate in vivo binding of GATA1 and C/EBPϵ at known GATA1 regulatory elements in primary eosinophils. GATA1 colocalizes with C/EBPϵ in a *cis*-element–specific manner, suggesting it may participate in different protein complexes on DNA. At both the intron element and IE promoter, GATA1 and C/EBPϵ colocalization raises the possibility that they may cooperate and physically interact. In C/EBPϵ-null mice, eosinophil differentiation defect is not as early as that seen with GATA1 loss.[10,22] Although this may suggest that eosinophil GATA1 expression is not C/EBPϵ dependent, eosinophil GATA1 levels in C/EBPϵ-null mice have not been reported. If there was a partial reduction in eosinophil GATA1 expression, this may account for some (or all) of the eosinophil phenotype in C/EBPϵ-null mice.

Taken together, unperturbed eosinophil GATA1 mRNA expression and eosinophil differentiation in mice lacking HS 1, weak enrichment for acetylated H3/H4 at HS 1 in primary eosinophils, and absence of regulator binding at HS 1 suggest that HS 1, in its normal chromosomal context, is not important for mouse eosinophil GATA1 expression. In contrast, our data show for the first time that the intron element is likely to function in regulating eosinophil GATA1 expression.

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References

1. Iwasaki H, Mizuno S, Wells RA, Cantor AB, Watanabe S, Akashi K. GATA-1 converts lymphoid and myelomonocytic progenitors into the megakaryocyte/erythrocyte lineages. Immunity. 2003; 19:451-462.
2. Kulessa H, Frampton J, Graf T. GATA-1 reprograms avian myelomonocytic cell lines into eosinophils, thromboblasts, and erythroblasts. Genes Dev. 1995;9:1250-1262.
3. Heyworth C, Pearson S, May G, Enver T. Transcription factor-mediated lineage switching reveals plasticity in primary committed progenitor cells. EMBO J. 2002;21:3770-3781.
4. Cantor AB, Orkin SH. Hematopoietic development: a balancing act. Curr Opin Genet Dev. 2001;11:513-519.
5. McNagny K, Graf T. Making eosinophils through subtle shifts in transcription factor expression. J Exp Med. 2002;195:F43-47.
6. McDevitt MA, Fujiwara Y, Shivdasani RA, Orkin SH. An upstream DNaseI hypersensitive region of the hematopoietic-expressed transcription factor GATA-1 gene confers developmental specificity in transgenic mice. Proc Natl Acad Sci U S A. 1997; 94:7976-7981.
7. Onodera K, Takahashi S, Nishimura S, et al. GATA-1 transcription is controlled by distinct regulatory mechanisms during primitive and definitive erythropoiesis. Proc Natl Acad Sci U S A. 1997;94:4487-4492.
8. Shivdasani RA, Fujiwara Y, McDevitt MA, Orkin SH. A lineage-selective knockout establishes the critical role of transcription factor GATA-1 in megakaryocyte growth and platelet development. EMBO J. 1997;16:3965-3973.
9. McDevitt MA, Shivdasani RA, Fujiwara Y, Yang H, Orkin SH. A "knockdown" mutation created by cis-element gene targeting reveals the dependence of red blood cell maturation on the level of transcription factor GATA-1. Proc Natl Acad Sci U S A. 1997;94:6781-6785.
10. Yu C, Cantor AB, Yang H, et al. Targeted deletion of a high-affinity GATA-binding site in the GATA-1 promoter leads to selective loss of the eosinophil lineage in vivo. J Exp Med. 2002;195:1387-1395.
11. Fujiwara Y, Chang AN, Williams AM, Orkin SH. Functional overlap of GATA-1 and GATA-2 in primitive hematopoietic development. Blood. 2004;103:583-585.
12. Hirasawa R, Shimizu R, Takahashi S, et al. Essential and instructive roles of GATA factors in eosinophil development. J Exp Med. 2002;195:1379-1386.
13. Dent LA, Strath M, Mellor AL, Sanderson CJ. Eosinophilia in transgenic mice expressing interleukin 5. J Exp Med. 1990;172:1425-1431.
14. Ishida Y, Levin J, Baker G, et al. Biological and biochemical characteristics of murine megakaryoblastic cell line L8057. Exp Hematol. 1993;21:289-298.
15. Johnson KD, Grass JA, Boyer ME, et al. Cooperative activities of hematopoietic regulators recruit RNA polymerase II to a tissue-specific chromatin domain. Proc Natl Acad Sci U S A. 2002;99: 11760-11765.
16. Bulger M, Sawado T, Schubeler D, Groudine M. ChIPs of the beta-globin locus: unraveling gene regulation within an active domain. Curr Opin Genet Dev. 2002;12:170-177.
17. Johnson KD, Bresnick EH. Dissecting long-range transcriptional mechanisms by chromatin immunoprecipitation. Methods. 2002;26:27-36.
18. Elefant F, Cooke NE, Liebhaber SA. Targeted recruitment of histone acetyltransferase activity to a locus control region. J Biol Chem. 2000;275: 13827-13834.
19. Tsai S-F, Strauss E, Orkin SH. Functional analysis and in vivo footprinting implicate the erythroid transcription factor GATA-1 as a positive regulator of its own promoter. Genes Dev. 1991;5:919-931.
20. Kobayashi M, Nishikawa K, Yamamoto M. Hematopoietic regulatory domain of gata1 gene is positively regulated by GATA1 protein in zebrafish embryos. Development. 2001;128:2341-2350.
21. Vyas P, McDevitt MA, Cantor AB, Katz SG, Fujiwara Y, Orkin SH. Different sequence requirements for expression in erythroid and megakaryocytic cells within a regulatory element upstream of the GATA-1 gene. Development. 1999;126:2799-2811.
22. Yamanaka R, Barlow C, Lekstrom-Himes J, et al. Impaired granulopoiesis, myelodysplasia, and early lethality in CCAAT/enhancer binding protein epsilon-deficient mice. Proc Natl Acad Sci U S A. 1997;94:13187-13192.
23. Zhang DE, Zhang P, Wang ND, Hetherington CJ, Darlington GJ, Tenen DG. Absence of granulocyte colony-stimulating factor signaling and neutrophil development in CCAAT enhancer binding protein alpha-deficient mice. Proc Natl Acad Sci U S A. 1997;94:569-574.