4-15-2010

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The Journal of Immunology

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J Immunol 2010; 184:4349-4361; Prepublished online 19 March 2010;
doi: 10.4049/jimmunol.0900927
http://www.jimmunol.org/content/184/8/4349

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

PU.1 Positively Regulates GATA-1 Expression in Mast Cells

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Coexpression of PU.1 and GATA-1 is required for proper specification of the mast cell lineage; however, in the myeloid and erythroid lineages, PU.1 and GATA-1 are functionally antagonistic. In this study, we report a transcriptional network in which PU.1 positively regulates GATA-1 expression in mast cell development. We isolated a variant mRNA isoform of GATA-1 in murine mast cells that is significantly upregulated during mast cell differentiation. This isoform contains an alternatively spliced first exon (IB) that is distinct from the first exon (IE) incorporated in the major erythroid mRNA transcript. In contrast to erythroid and megakaryocyte cells, in mast cells we show that PU.1 and GATA-2 predominantly occupy potential *cis*-regulatory elements in the IB exon region in vivo. Using reporter assays, we identify an enhancer flanking the IB exon that is activated by PU.1. Furthermore, we observe that in PU.1$^{-/-}$ fetal liver cells, low levels of the IE GATA-1 isoform is expressed, but the variant IB isoform is absent. Reintroduction of PU.1 restores variant IB isoform and upregulates total GATA-1 protein expression, which is concurrent with mast cell differentiation. Our results are consistent with a transcriptional hierarchy in which PU.1, possibly in concert with GATA-2, activates GATA-1 expression in mast cells in a pathway distinct from that seen in the erythroid and megakaryocytic lineages. ***The Journal of Immunology*****, 2010, 184: 4349–4361.**

Mast cells are central effectors in the pathogenesis of allergic and inflammatory disorders, and they also participate in normal host defense (1–3). Mature mast cells in the connective and mucosal tissues differentiate from uncommitted hematopoietic stem cells in the bone marrow; this process of lineage selection is orchestrated by a network of tightly regulated transcription factors (4). Current models of hematopoiesis suggest that multiple lineage-specific transcription factors are expressed at low levels in early, pluripotent progenitor cells. During differentiation, subsets of these transcription factors become dominantly expressed in a lineage-restricted fashion. It is increasingly recognized that antagonistic programs provide decision trees through binary switches and more complex antagonistic pathways. Activation of one program leads to antagonism and repression of the other. One example is the relationship between the GATA-1 and PU.1 transcription factors. GATA-1 is an essential transcriptional regulator for the erythroid and megakaryocyte lineages (5–8), whereas it is absent in neutrophils and monocytes. GATA-1 requires interaction with the cofactor FOG-1 for erythroid and megakaryocyte development. However, FOG-1 has been shown to antagonize mast cell development (9, 10), and it inhibits the expression of GATA-1–dependent mast cell-specific genes (11). Alternatively, PU.1 is a critical transcription factor for neutrophils and monocytes (12, 13), but it is downregulated during erythroid differentiation. Reciprocal activation of GATA-1 and PU.1 in early, multipotent progenitors restricts differentiation potential to either the megakaryocyte/erythroid or lymphoid/myeloid lineages, respectively (14, 15). Furthermore, these two factors have been shown to be antagonistic in monocytic and erythroid cells; however, both GATA-1 and PU.1 are required for the normal development of the mast lineage (16, 17).

The mechanisms by which these factors are coexpressed and might cooperate in mast cell differentiation are poorly understood. A number of studies have demonstrated the functional antagonism between PU.1 and the GATA factors. Forced expression of GATA-1 into myelomonocytic cells reprograms their differentiation to erythroid cells, eosinophils, or megakaryocytic precursors (18, 19). Conversely, PU.1 overexpression represses erythroid differentiation (20, 21). The mechanism of antagonism appears to be due to direct interactions between the DNA binding domains of these proteins. GATA-1 has been shown to inhibit transcriptional activity of PU.1 through direct physical interactions that result in displacement of the PU.1 coactivator c-Jun (22, 23). PU.1 appears to interfere with the expression of GATA-1–dependent targets by disrupting of the ability of GATA-1 to bind DNA (23). Although these two factors are antagonistic, they are coexpressed at low levels in multipotential hematopoietic precursors in what can be considered a priming stage (24). During lineage selection to either myeloid or erythroid, PU.1 or GATA-1 is upregulated selectively to become the dominantly expressed factor in the respective lineages. Mast cells are unique in that both PU.1 and GATA-1 are coexpressed and required for proper maturation. This suggests that unique mast cell-specific mechanisms are in play to regulate these factors.

GATA-1 expression is controlled by highly conserved regulatory elements. Two well-characterized mRNA isoforms of GATA-1

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Received for publication March 24, 2009. Accepted for publication February 11, 2010.

This work was supported by the Basil O'Conner Starter Scholar Research Award Grant 5-FY04-30 from the March of Dimes Birth Defects Foundation (to C.M.T.), a Children's Cancer Foundation grant (to C.M.T.), the National Institutes of Health Grant 5R01HL077178 (to C.M.T.), the Johns Hopkins Malaria Research Institute and the Bloomberg Family Foundation Grant (to M.A.M.), and U.S. Department of Defense Grant 0510343 (to M.A.M.).

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The online version of this article contains supplemental material.

Abbreviations used in this paper: BMMC, bone marrow-derived mast cell; ChIP, chromatin immunoprecipitation; HS IE, hypersensitive site upstream of the IE exon; HS IB, hypersensitive site in IB exon region; HS UE, hypersensitive site upstream enhancer; IBds, genomic region downstream of the IB exon; IEP, IE promoter; G1, GATA-1; G2, GATA-2; UE, upstream enhancer.

arise from alternatively spliced noncoding first exons that are under the control of tissue-selective promoters: the promoter proximal to the IE exon is active in hematopoietic cells (25), and the IT promoter is primarily used in the testis (26). The IE GATA-1 isoform appears to be the major isoform in hematopoietic cells; the expression of this isoform in erythroid cells and megakaryocytes is dependent on hypersensitive regions upstream of the transcriptional start site (27, 28). Eosinophils also express and require GATA-1 for normal development. A targeted deletion of a region upstream of the IE exon, which contains a highly conserved GATA binding site palindrome, results in a selective deficiency of eosinophils (29), while mast cells, erythroid cells, and megakaryocytes are spared. In contrast, deletion of first enhancer (hypersensitive site I) and distal promoter of the GATA-1 gene leads to abnormal erythroid, megakaryocyte, and mast cell development, but eosinophil differentiation is not affected (30). The existence of cell-specific enhancers and/or mRNA isoforms allows for cell-specific expression of these transcription factors; however, the regulatory elements that control GATA-1 expression in mast cells have not been explored.

In addition to GATA-1, a number of transcription factors have been demonstrated to play essential roles in mast cell development. PU.1 is an ETS family transcription factor that is a critical regulator of the myeloid and lymphocyte lineages. Mouse embryos with a targeted deletion of PU.1 lack skin mast cells (16). Furthermore, fetal liver cells deficient in PU.1 cannot differentiate into the earliest mast cell precursors. Restoration of mast cell differentiation is dependent on the expression of PU.1 and GATA-2 (16). GATA-2 is a zinc finger transcription factor similar to GATA-1. Murine embryonic stem cells deficient in GATA-2 do not differentiate into mast cells, illustrating its requirement for early commitment to the mast lineage (31). In contrast to GATA-2 and PU.1, GATA-1 appears important for terminal differentiation. The hypomorphic GATA-1 low mutation (deletion of upstream enhancer and distal promoter) results in increased proliferation and impaired differentiation of mast cell progenitors (17, 32). The different developmental phenotypes of these mutations suggest that these transcription factors act at different stages of mast cell differentiation.

The goal of this study was to investigate the regulation of GATA-1 in mast cells. We identified a unique mRNA isoform of GATA-1 that is abundantly expressed during mast cell development. Based on the requirement of PU.1 and GATA-2 for early mast cell development, we speculated that these factors might participate in the regulation of GATA-1 in mast cells. Using a computational approach (33), we identified consensus GATA and PU.1 binding sites within phylogenetically conserved regions of the GATA-1 gene. We determined whether the GATA and PU.1 factors bound these putative sites in vivo and ex vivo with chromatin immunoprecipitation (ChIP) and EMSA, respectively. Potential regulatory regions were examined with reporter assays. We examined the physiologic requirement of PU.1 for the IB GATA-1 isoform expression in mast cells using PU.1-deficient fetal liver cells and show an absolute requirement for PU.1. This work demonstrates that PU.1 regulates GATA-1 expression in mast cells; both PU.1 and GATA-2 occupy mast cell specific *cis*-regulatory elements in the *GATA1* gene. The findings support a model for a transcriptional network regulating GATA-1 in mast cells that is distinct from that in erythroid cells and megakaryocytes.

Materials and Methods

Cells

Murine embryonic stem cells were differentiated into mast cells according to the method described previously (34). Strain 129v murine embryonic stem cells were grown on a feeder layer of mitomycin-treated mouse embryonic fibroblasts in DMEM (GIBCO, Grand Island, NY) supplemented with 15% ES grade FBS (HyClone, South Logan, UT), 1% penicillin/streptomycin and glutamine, 100 μM β mercaptoethanol, 1 mM sodium pyruvate, 1% nonessential amino acids (GIBCO) and 100 U/ml of mouse recombinant LIF (Chemicon International, Temecula, CA). Embryonic stem cells were adapted to IMDM (GIBCO) with supplements as listed above and grown on 0.2% gelatin coated plates prior to differentiation. To differentiate embryonic stem cells, 5000 cells were plated on bacterial-grade Petri dishes in 1.5 ml IMDM with 0.9% methylcellulose (StemCell Technologies, Vancouver, Canada) supplemented with 15% ES grade FBS, 1% penicillin/streptomycin and glutamine, 434 μM monothioglycerol, 1 mM sodium pyruvate, 1% nonessential amino acids, with 50 ng/ml SCF and 5 ng/ml of IL-11. After 1 wk, developing embryoid bodies were supplemented with culture media containing 60 ng/ml SCF, 30 ng/ml mouse recombinant IL-3, and 30 ng/ml IL-6. At week 2 of culture, embryoid bodies were transferred to tissue culture flasks and allowed to differentiate in DMEM supplemented with 10% FCS, 1% penicillin/streptomycin and glutamine, 20% WEHI supernatant, 50 ng/ml SCF. Mast cells were identified by their morphologic appearance on light microscopy. The percentage of differentiated mast cells was obtained by counting over 500 cells from 3 independent experiments. Bone marrow-derived mast cells (BMMCs) were derived from 4–8-wk-old wild type C57/BL6 mice. Mice were maintained in The Johns Hopkins University Animal Facilities in accordance with institutional guidelines. Bone marrow obtained from femurs and tibias and splenocytes obtained from spleens from these mice were cultured in DMEM with 10% FCS supplemented with 1% penicillin/streptomycin and glutamine as well as 20% WEHI supernatant and 50 ng/ml of mouse recombinant SCF as previously described (35). The HMC-1 human mast cell lines and the L8057 murine megakaryocytic cell lines were maintained in RPMI 1640 with 10% FBS and 1% penicillin/streptomycin and glutamine. The HeLa cell lines, the murine erythroleukemia MEL cell lines, and the NIH 3T3 murine fibroblast cell lines were maintained in DMEM with 10% FBS and 1% penicillin/streptomycin and glutamine. The C57 murine mast cell line was maintained in DMEM with 10% FBS and 1% penicillin/streptomycin and glutamine and 5×10^{-5} M β mercaptoethanol. The PU.1$^{-/-}$ fetal liver cells were described previously (16). These cells are maintained in RPMI 1640 (GIBCO) supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO), 1% penicillin/streptomycin and glutamine (GIBCO), and 100 μM β mercaptoethanol with 5 ng/ml of recombinant murine IL-3.

Plasmids

The pMMP-IRES-puro retroviral vector has been described previously (34). pMMP-IRES-puro PU.1 was constructed by subcloning the PU.1 cDNA into the pMMP-IRES-puro backbone. The pEBB expression constructs have been described previously (35). pEBB GATA-1, pEBB GATA-2, and pEBB PU.1 were constructed by subcloning the cDNAs for GATA-1, GATA-2, and PU.1 into the pEBB backbone. The inserts were amplified by PCR with an Nde site engineered at the 5′ end and a Cla1 site engineered at the 3′ end. The reporter assays were constructed by cloning genomic fragments of the GATA-1 locus into the xho1 kpn1 site of the pGL2 pro vector. Mutations in the PU.1 binding site were created with the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to manufacturer's recommendations. The core GGAA sequences were mutated to TCGC. The primers used to amplify by PCR the genomic fragments are as follows: pUE luciferase forward 5′-GTC AAA CAC ATG ACT CCT TGG T-3′, reverse 5′-GGT GCT GGA CTC ATA TCC CAT-3′: pIEP luciferase forward 5′-CCC CAG CCC CAA GAC AGC CT-3′, reverse 5′-GTC TCC CTT CTC TCC CTC CTG CCA-3′: pGP2 luciferase forward 5′-CCA AAC CCC AAA CAG ATC TC-3′, reverse 5′-GGC CTG GAC TTC TCA CCT TT-3′: pIBb luciferase forward 5′-GCA GTG TGG GGG CAG GAG-3′, reverse 5′-GGC AGA ATG CAG GAC CAA G-3′: pIBa luciferase forward 5′-AGT GGT GAG ACT CAA AGG-3′, reverse 5′-GGC AGA ATG CAG GAC CAA G-3′: pIBds luciferase forward 5′-CTT GGT CCT GCA TTC TGC CT-3′, reverse 5′-TGT GTG TCT CCT TTT GGG C-3′: phu IBds luciferase forward 5′-TCT GGT CCT GCA TGC CAT CCC-3′, reverse 5′-TCC TTT TTC TCC TCC ACC TTC-3′.

Retroviral transduction of PU.1 fetal liver cells

Retrovirus was produced using the method described by Ory et al. (36). The 293GPG cell line was grown in 15-cm tissue culture plates with DMEM supplemented with 10% FBS, 1% penicillin/streptomycin and glutamine, and 1 μg/ml doxycycline. The 293GPG cell line was transfected with 25 μg pMMP-IRES-puro-PU.1 and 75 μg FuGENE 6 (Life Technologies). Medium was replaced daily and doxycycline was removed to induce expression of VSV-G. Supernatant was collected on days 4–8 after transfection. Retrovirus was concentrated by centrifuging supernatant at 26,000 × *g* in

an ultracentrifuge for 1.5 h. Concentrated retrovirus was resuspended in TEN buffer overnight at 4°C. PU.1 fetal liver cells were transduced with concentrated retrovirus (100–200 μl) with 8 μg/ml polybrene for ~24 h. Retroviral transduction was repeated twice, after which cells were resuspended in fresh media with IL-3 at 5 ng/ml and SCF at 50 ng/ml. After 2–3 d in culture, 2 μg/ml puromycin was added and maintained in culture to select for stably transduced cells.

Real-time quantitative PCR

RNA was harvested from cells with Trizol (Invitrogen), and cDNA was made with the first-strand synthesis kit (Invitrogen); 1–5 μg RNA was used as starting material for synthesis. Real-time quantitative PCR was performed using a SYBR green mix (BioRad, Hercules, CA) on an iQ5 multicolor real-time PCR detection system (Bio-Rad, Hercules, CA). Primers were optimized for temperature and were used at a concentration of 200 nM. The primers sequences are as follows: IE GATA-1 forward 5′-CCC TGA ACT CGT CAT ACC ACT AAG-3′, IB GATA-1 forward 5′-AAA CTC CTA TGG GAG CTG TCA AGG-3′: GATA-1 common forward 5′-ATC AGC ACT GGC CTA CTA CAG AG-3′, GATA-1 reverse 5′-GAG AGA AGA AAG GAC TGG GAA AG-3′: PU.1 forward 5′-AGA GCA TAC CAA CGT CCA ATG C-3′: PU.1 reverse 5′-GTG CGG AGA AAT CCC AGT AGT G-3′: GATA-2 forward 5′-CAA GAA AGG GGC TGA ATG TTT CG-3′: GATA-2 reverse 5′-GTG TCC CAC AGG TGC CAT G-3′: β actin forward 5′-GTG ACG AGG CCC AGA GCA AGA G-3′: β actin reverse 5′-AGG GGC CGG ACT CAT CGT ACT C-3′. Conventional PCR primers and conditions for IE GATA-1, IB GATA-1, GATA-1 common, GATA-2, PU.1, and β actin are the same used for real-time quantitative PCR. Relative quantification of mRNA of GATA-1, GATA-2, and PU.1 between the mast cell line, erythroid line, megakaryocyte line, and fibroblast line was determined by taking the ratio of the signal divided by the signal of β actin from that cell line, and then scaling to a mast cell signal of 1. For the GATA-1 IE and IB relative signals, the ratio of the β actin normalized GATA-1 IE or IB signals to the β actin normalized GATA-1 common signal was determined.

Scanning chromatin immunoprecipitation was performed as previously described (37). The name of the ChIP primers denotes the position in kb relative to the GATA-1E start site. Primer sequences are listed as follows: −42 forward 5′-ACA GAA CTA TCC ATT GGA CCA AAC T-3′, −42 reverse 5′-ACC ACA TCC ACA CAG TCA GTA AAA G-3′: −32.5 forward 5′-GCG TGC CCC ATG TCT CA-3′, −32.5 reverse 5′-GGC TAA AAA GCT TGG CAC ACA-3′: −25 forward 5′-TGC ATG CAT TTC CCT AAC TAC TGT-3′, −25 reverse 3′-CCT CCA TCG CTT TAC TGT CAC TT-3′: −4 (HS UE) forward 5′-CCT GCT GGC TGA ACT GTG-3′, 4 (HS UE) reverse 3′-TGG TGT GGT GCT GGA CTC-3′: 0 (HS IE) forward 5′-AAA GAA GTG TAT GTA CCC TTA CCC-3′, 0 (HS IE) reverse 5′-CGT GAG CCC TCC TGA ATG C-3′: +2 5′-TCC TTC CCT TAA ACT CCA CAG C-3′, +2 reverse 5′-TGG TAG TTA GGA GGT TAG AGG TAG-3′: +3.5 (HS IB) forward 5′-AGT CAG ATG GCA AGA TAC AAC AG-3′, reverse +3.5 (HS IB) 3′-TCC TCC TTT ACT TTC CTT CTA ACC-3′: +8 forward 5′-GGT TAC ACC CCT GTC TTC CTC C-3′, +8 reverse 5′-GCT CAC AAG AGA TCC ACC TGC-3′: +19 forward 5′-CCC ATT CAG GAG ACA CTG TCC-3′, reverse +19 5′-GTC AGA AAG CCC GAC TTT CG-3′: +38 5′-ACA GTG CGT GAC ATC TCA AGC-3′, +38 reverse 5′-GTG GCC CAA TGT TCT GAC C-3′. Cycling parameters were for quantitative PCR was as follows: 95°C for 30 s, 50°–65°C for 30 s, 72°C for 30 s. Forty cycles were performed.

5′ RACE

mRNA was isolated from the C57 mast cell line and the M1 myeloid cell line using the mRNA isolation kit (Stratagene). 5′RACE was performed using the SMART RACE cDNA amplification kit (BD Biosciences, San Jose, CA) according to manufacturer's recommendations. The 3′ primer used for amplification of 5′ RACE products was CGC TCC GTC TTC AAG GTG TCC AAG AAC G.

RNase protection assay

Total RNA was extracted from the C57 mast cell line with Trizol (Invitrogen). RNase protection assays were performed using the RiboQuant RPA kit (BD Biosciences) according to manufacturer's recommendations. The RNA antisense probe used to determine the transcriptional initiation site was made with the RiboQuant In Vitro Transcription Kit (BD Biosciences, Mississauga, Ontario, Canada). The template for in vitro transcription was constructed by PCR amplification of the genomic sequence of the GATA-1 intron and subcloning into the pCR2.1 (Invitrogen) vector. The 5′ primer for PCR was upstream of the IB exon (GAT AAA GCC GGA AAC CAC GGG) and the 3′ primer was within the IB exon (CGG CAA GCC TTT GCT TCT CTT TCT), which amplified a 243-bp product. The resultant RNA probe was 311 bp because of an additional 68 bp of vector sequence.

EMSAs

Nuclear extracts were extracted from the mast cell line C57 and from COS cells transfected with the expression constructs pEBB GATA-1, pEBB GATA-2, pEBB PU.1, and pEBB vector. COS cells were transfected using the DEAE-dextran method as previously described (35); 5 μg DNA was mixed with a 2.5 mM chloroquine and 0.1% DEAE-dextran solution and added to 5 ml DMEM with 10% FBS. The mixture was added to a 10-cm plate of COS cells for 4 h at 37°C, and the media was replaced with 10% DMSO in PBS for 2 min at room temperature. The solution was removed and fresh media were added. Cells were harvested after 72 h, and nuclear extracts were obtained as described (38). Double-stranded oligonucleotide probes were synthesized and are as follows with the GATA and PU.1 binding sequences underlined: UE G 5′-GCC CCC GCT GAT TCC CTT ATC TAT GCC TTC CCA GC-3′; UE- 5′-ACA GGG ATG GGG GAG GGA ATG GGG TGA GGC CTG TC-3′; IE GG 5′-GAG TCC ATC TGA TAA GAC TTA TCT GCT GCC CCA G-3′; GI GG 5′-CCA GCC TGG AGA TAA ACT TTA TCT CTG TCC GGA-3′; IB- 5′-GGG TGA TTT CAA AAG TTG GGC GGG GAT GGC AGA GA-3′; IB G1 5′-GCG GGG ATG GCA GAG ATA AGC AGT GTG GGG GCA-3′; IB G2 5′-CAA GAT ACA ACA GAT AGG GAT GAA GTT GGG GAG CA-3′; Ms IB P1 5′-TTA CTC TTC TCA GAG GAA GCC AGT AGC TTT GG-3′; Ms IB P2 5′-CCA TAG AAC AGG GAG GAA CTA ACG GGG CCC ACA-3′; Ms IB P3 5′-GAA GAG AGA ATA AAA AGA GGA AAT GGA GGA GGC CTT-3′; Ms IB P4 5′-AGG CTT GCC GGA GGA GGA GAA AGA GGA AGG TTA GAA GGA-3′; Ms IB P5 5′-GAG GAG ACA CAG AAA GAG GAA GAG AAA TAT GGT AC-3′; Ms IB P6 5′-CAA GAC AGT GGA AAG AGG AAA AGG GGA AGA AAA-3′; Hu IB P3 5-AGA ATA AGA GGA AGT GGA GGA GG-3′; Hu IB P5 5′-GTA GAC AGG GGA AGA GAG GCC-3′. For PU.1-mutated probes, the GGAA core sequence was changed to TCGC. Oligonucleotide probes were end-labeled with T4 polynucleotide kinase (New England Biolabs, Beverly, MA) with γ[^{32}P] ATP according to manufacture's instructions. Gel shift analysis was performed as previously described (35). Reactions were performed in 20 μl volumes with 5% glycerol, 100 mM KCl, 10 mM Tris-Cl (pH 7.4), 1 mM dithiothretol, 0.3 μg of poly(dI-dC), and ~5 × 10^5 cpm of [^{32}P]-end labeled oligonucleotide probe. Supershift analysis was done with 4 μg Ab against GATA-1 (N6 clone), GATA-2 (H-116 clone), and PU.1 (T-21 clone). All Abs were from Santa Cruz Biotechology, Santa Cruz CA and at a concentration of 200 μg/0.1 ml. For no-Ab controls, 4 μg BSA was used. The reactions were separated on 4.5% PAGE with 0.5× TBE with 0.1% glycerol as buffer.

Reporter assays

Transfections of the HMC-1 human mast cell line were performed with the Superfect reagent (Qiagen, Valencia, CA) according to the manufacturer's recommendation. Briefly, 3 × 10^5 cells were placed in 1500 μl RMPI 1640 with 10% FBS in 24-well plates; 4 μl Superfect solution was added to 2 μg DNA to make a total volume of 100 μl in OptiMEM. The drive-to-reporter ratio was 1:4. Sea pansy luciferase 0.05 μg was added to the Superfect: DNA mixture for normalization and the mixture was allowed to incubate at room temperature for 15 min; 1200 μl RPMI with 10% FBS was added to the mixture and then added to the cells. Cells were incubated overnight at 37°C and lysates were harvested the following day in 150 μl of passive lysis buffer (Promega, Madison, WI). Transfections of the HeLa cell line were performed with the Effectene Transfection reagent (Qiagen) according to manufacturer's recommendation. Briefly, 1 × 10^5 cells were plated in 500 μl DMEM with 10% FBS in 24-well plates. A total of 0.5 μg DNA was used at DNA-to-reagent ratio of 1:25. The driver-to-reporter ratio was 1:4. Sea pansy luciferase (0.1 μg) was used for normalization. Cells were incubated overnight with the DNA:Effectene complexes at 37°C, and lysates were harvested the following day in 150 μl passive lysis buffer (Promega). For both HMC-1 and HeLa transfections, 20 μl lysate was assayed for luciferase activity using the dual luciferase system (Promega). All experiments were done in triplicate and normalized to sea pansy luciferase activity.

Results

An alternative GATA-1 isoform is expressed in murine mast cells and myeloid cells

As the first step in characterizing the potential regulation of GATA-1 in mast cells, we performed 5′RACE in a well-characterized murine mast cell line C57. Two abundant RACE products were

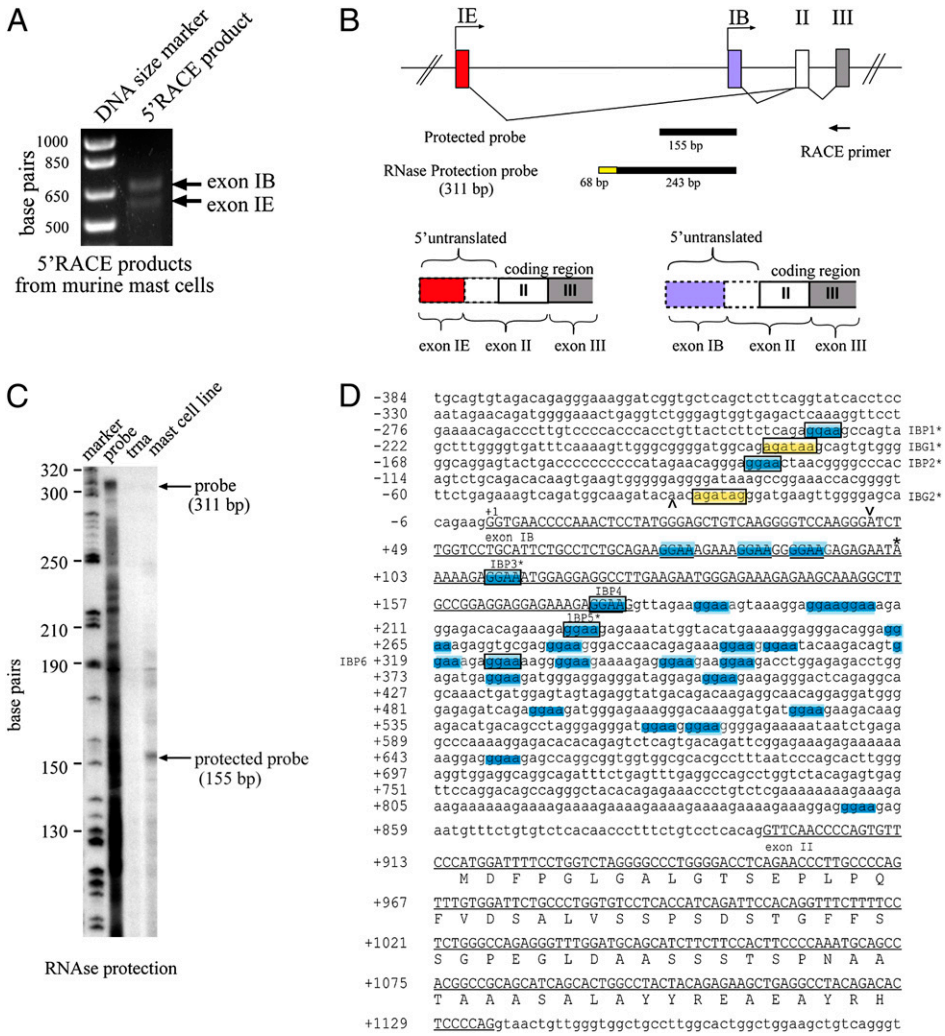

FIGURE 1. A variant mRNA isoform of GATA-1 is expressed in murine mast cells. *A*, Abundant 5′RACE products of GATA-1 from the murine mast cell line C57. Products were separated on a 1.2% agarose gel and stained with ethidium bromide. Sequence analysis revealed that the ~600-bp product was the exon IE containing isoform of GATA-1, and the ~700-bp product was the exon IB containing isoform. *B*, Schematic representation of the IE and IB isoforms. These isoforms arise for a differential splicing event that places the untranslated first exon onto exon II. The translational start ATG lies within exon II for both isoforms. The RNase probe contains 243 bp of sequence from the IB exon (black bar) and an additional 68 bp of vector sequence (yellow bar). The resultant probe length was 311 bp. The major protected probe is 155 bp in length. The 3′RACE primer lies within exon III. *C*, RNase protection assay demonstrating multiple protected bands in the C57 murine mast cell line. The total probe length is 311 bp in length (243 bp of exon IB sequence plus 68 bp of vector sequence). The major protected probe size is 155 bp long (arrow). *D*, Sequence of GATA-1 isoform identified by 5′RACE from murine mast cells. The transcriptional start site was determined by RNase protection (based on the major probe fragment of 155 bp) and denoted with a +1. The initial nucleotide of the most common transcript represented in sequenced RACE products is denoted with a *v* (above nucleotide) and extends 135 bp upstream of exon 2. The initial nucleotide of the longest transcript represented in sequenced RACE products is denoted by ^ (below nucleotide) and extends 212 bp upstream of exon 2. A shorter transcript described previously by Seshasaye et al. (39) is shown with an * (above nucleotide) and extends 78 bp upstream of exon 2. The exon IB and exon II are underlined and capitalized. An open reading frame begins in exon II and the translated amino acids are depicted. Consensus GATA sites are highlighted in yellow and PU.1 sites (GGAA) are highlighted in blue. Consensus binding sites that are contained within oligonucleotide EMSA probes are boxed and labeled. EMSA probes containing consensus sites that are conserved between mouse and human are denoted by an *. IBP1, IBP2, IBP3, IBP4, IBP5, IBP6 refer to EMSA probes with a PU.1 binding motif. IBG1 and IBG2 refer to EMSA probes with a GATA binding motif.

amplified and sequenced: a ~600-bp product and a larger ~700-bp product (Fig. 1*A*). Analysis of the sequences demonstrated that the smaller product was the isoform of GATA-1 containing the IA exon found predominantly in erythroid cells; the larger product, however, was an isoform of GATA-1 that contained a distinct exon. Sequence analysis of ~30 independently derived 5′ RACE products demonstrated that this isoform incorporated a noncoding first exon that was alternatively spliced onto exon II. Previous work has described a similar exon IB (25), initially identified in a rare transcript expressed in erythroleukemia cells. Additional studies showed an exon IB-containing transcript in primary murine myeloid colony cells (39) and eosinophils (40). These larger transcripts that we identified by RACE are compared with previously identified transcripts in Fig. 1*D*. The IB exon lies 3.7 kb downstream of the IE exon and is in close proximity to a previously identified hypersensitive site (41). Sequence analysis of multiple C57 5′RACE products demonstrates that the complete IB exon in mast cells is considerably longer than what was previously described. On inspection of the sequences upstream of IB, no TATA boxes were identified. The 3′ end of IB was invariant with a splice donor site 736 bp upstream of the major GATA-1 AUG codon. We also identified the IB isoform in the myeloid cell line, M1, a murine leukemia cell line (42) by 5′RACE (data not shown). 5′RACE products of variable lengths were identified,

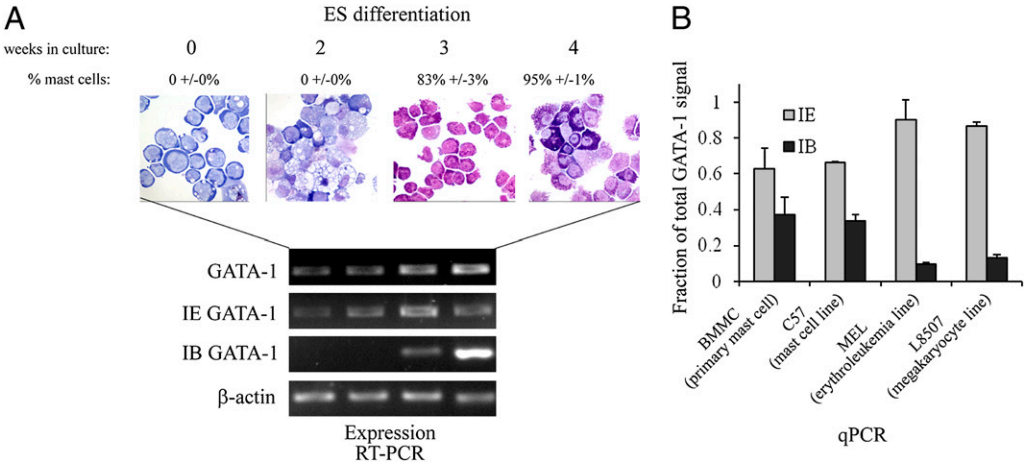

FIGURE 2. The IB GATA-1 isoform is upregulated during mast cell differentiation. *A*, Murine embryonic stem cells were differentiated in vitro, and the expression of GATA-1 isoforms was determined by RT-PCR. The IE GATA-1 isoform can be detected in undifferentiated cells and is sustained during differentiation. The IB GATA-1 isoform can be detected only when morphologically identifiable mast cells appear. Cytospin preparations of differentiated embryonic stem cells were stained with Wright-Giemsa. Quantification of mast cell differentiation was done by visual identification of cells expressing metachromatic granules stained with Wright-Giemsa. Photomicrograph images were acquired with a Kontron ProgRes 3012 digital camera and Roche Image analysis software with a Zeiss Axiophot microscope. Original magnification ×40. B, Relative expression of GATA-1 isoforms in cells expressing GATA-1. Quantitative PCR with SYBR green was performed in a murine mast cell line (C57), primary BMMCs, a murine megakaryocyte line (L8057), and a murine erythroleukemia line (MEL). Fraction of IE and IB isoform was calculated by taking the IE or IB signal and dividing by the sum of the IE and IB signals. Signals were normalized to β actin. IB GATA signal is detected from all cell types, but mast cells express significantly more IB GATA relative to total GATA-1.

suggesting possible heterogeneity in the transcriptional start site, as has been described for the IE exon (25). To delineate the 5′ end of the IB exon, we used RNase protection. As shown in Fig. 1*C*, we found multiple products that were protected in mast cells, with a predominant fragment (155 bp in length) that mapped a transcriptional start site to 179 bp upstream of exon 2 (Figs. 1*B–D*). We conclude that the multiple bands represent the presence of variable transcript lengths and possible probe degradation.

We examined the tissue-specific expression of this isoform during mast cell development with RT-PCR using isoform specific primers. Murine embryonic stem cells were differentiated in vitro in culture conditions suitable for mast cell differentiation (43), and RNA was harvested at various time points during the culture process. As shown in Fig. 2*A*, the IE exon-containing isoform of GATA-1 can be detected throughout development. However, the IB exon-containing isoform of GATA-1 can be detected only after week 3 of culture, when >80% of the cells are morphologically identifiable mast cells (Fig. 2*A*). We also assessed by quantitative PCR the relative expression of the GATA-1 isoforms in other cell types that express GATA-1. As shown in Fig. 2*B*, the IE exon-containing GATA-1 isoform is expressed in erythroid cells (MEL cell line), megakaryocytes (L8057 cell line), and mast cells (C57 cell line and primary BMMCs). However, the fraction of the IB exon-containing isoform comprises a significantly greater percentage of the total GATA-1 signal in mast cells (34% for C57 and 37% for primary mast cells) compared with erythroid (10%) and megakaryocyte (13%) lines (Fig. 2*B*).

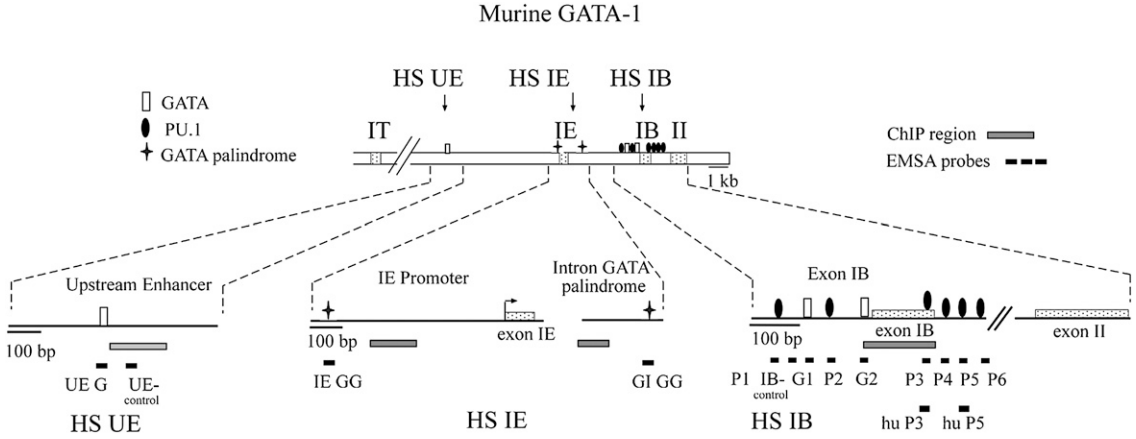

FIGURE 3. Putative transcription factor binding sites for GATA and PU.1 in the murine GATA-1 locus. Three genomic regions conserved between the mouse and human are depicted in the murine GATA-1 locus: the upstream enhancer, the exon IE promoter, the GATA palindrome within the first intron, and the hypersensitive site in proximity to exon IB. These sites correspond to previously identified DNaseI hypersensitive sites (labeled HS UE, HS IE, HS IB; see text for details). Putative GATA sites are denoted by rectangles, and PU.1 sites are denoted by black ovals. The GATA palindromic sites are depicted by stars. The regions spanned by PCR primers for ChIP are shown with gray bars. Sites of the EMSA probes used in Fig. 4 are depicted at the bottom of the figure and the denoted by black bars. The UE and IB control probes do not have consensus PU.1 or GATA binding sequences. dnhuP3 and huP5 denote EMSA probes from human sequence that are homologous to the murine corresponding murine probes (msP3 and msP5, respectively). Exons are shown by stippled bars.

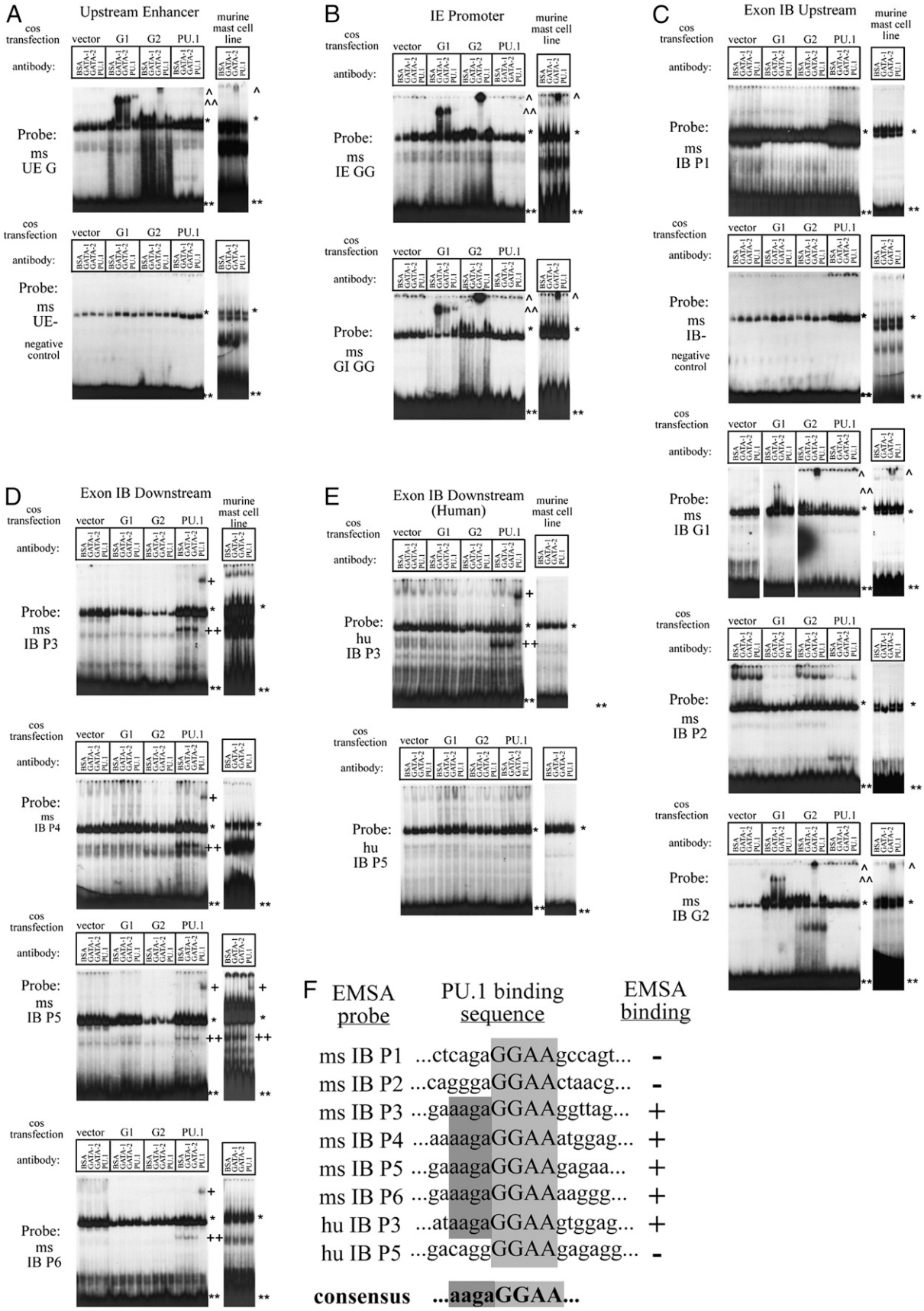

FIGURE 4. EMSAs demonstrate GATA-2 and PU.1 binding to consensus binding sites. Regions of EMSA probes are denoted in Figs. 1*D* and 3. COS cells were transfected with GATA-1, GATA-2, PU.1, or vector alone. EMSAs were performed with nuclear extracts from transfected COS cells and C57 mast cells with [^{32}P]-labeled oligonucleotide probes containing consensus DNA binding sites for transcription factors. Specific binding was determined by supershifting Abs. Specific GATA-2 complex is denoted by ^, and GATA-1 complex by ^^. PU.1 complexes are depicted by $^{+}$ and $^{++}$. A background band is denoted by *. The free probe is marked by **. *A*, GATA factor binding to the upstream enhancer region in vitro. GATA-1 and GATA-2 bind the UE G probe

Phylogenetic sequence comparisons identifies highly conserved PU.1 and GATA sites in the GATA-1 locus

To examine the potential transcriptional regulation of the IB containing GATA-1 isoform in mast cells, we first used TRANSFAC (www.gene-regulation.com/index.html) to inspect ~110,000 bp of human and murine GATA-1 sequences that compose the locus and found several hundred GATA and PU.1 sites, including those previously identified (27, 28, 41, 44). To focus the pool of possible sites to investigate, we used an independent analysis with the TRAFAC program (33), which has been used to identify conserved and functional Myc (37) and other transcription factor binding sites. Again, a number of consensus GATA and PU.1 sites were identified, although much fewer (Supplemental Data, Supplemental Fig. 1). This analysis in particular identified stretches of conserved sequences in close proximity to three previously identified hematopoietic-specific regulatory sites. These sites corresponded to previously determined DNase I-hypersensitive sites (Fig. 3, Supplemental Fig. 1). A high degree of conservation was identified ~3.5–4 kb upstream of the IE promoter. This region contains an upstream enhancer that has been demonstrated to be a DNase I-hypersensitive site in hematopoietic cells. It has been referred to previously as hypersensitive site I (44) and more recently HS-3.5 (41). For this study, we will refer to it as hypersensitive site upstream enhancer (HS UE). It contains a GATA binding site that is conserved between mouse and human and shown by mutation to be critical for expression of GATA-1 in transgenic hematopoietic tissues (44). Within the erythroid promoter, ~700 bp upstream of the IE exon, a stretch of highly conserved sequence coincides with another hypersensitive site, previously called hypersensitive site III (44) or IE (41). We refer to this as the hypersensitivity site upstream of the IE exon (HS IE) because of its proximity to the IE exon and promoter (Fig. 3). This region contains a highly conserved GATA palindromic binding sequence and other sequences necessary for expression of GATA-1 in eosinophils (29). In addition, we found another phylogenetically conserved region downstream of the IE exon that also contained a GATA palindrome binding site (Fig. 3, Supplemental Fig. 1). The IB exon mapped to a highly conserved region within the GATA-1 intron; this had been previously demonstrated to be a DNase I hypersensitive site, named HS+3.5 (41). We refer to the site as HS IB. Computational analysis identified several putative GATA and PU.1 binding sites within this area; the highest degree of phylogenetic conservation is found at the 5′ region of the IB exon. Two consensus GATA binding sites were identified upstream of the IB exon; these were both conserved in humans (Fig. 1*D*, labeled IBG1* and IBG2*, highlighted yellow). Two conserved putative PU.1 sites (labeled IBP1* and IBP2*, highlighted blue) were also found in this region. The 3′ flanking sequences of the IB exon contains a GA-rich region (45) with numerous potential PU.1 binding sites (Fig. 1*D*, highlighted blue); however, the degree of conservation between murine, rat and human is diminished. Within the IB exon and intron, two GGAA sites were conserved between mouse and human (IBP3* and IBP5*). Based on published role of GATA factors and PU.1 in mast cell development, we focused further studies on these conserved GATA and PU.1 sites as possible regulatory elements in mast cells.

GATA-2 binds the GATA-1 locus in vitro

Since prediction algorithms are only a first step in identifying functional elements, we used EMSA to examine the capacity for GATA factors and PU.1 to bind to the highly conserved putative DNA binding sites in the GATA-1 locus. To assess the DNA binding of each hematopoietic factor individually—without the potential influence of GATA factor/PU.1 complex formation—GATA-1, GATA-2, and PU.1 were individually expressed in COS cells, which do not express any of these factors. Specificity of binding was determined by supershifting Abs. Probes containing single conserved GATA sites from the upstream enhancer region (UE G) and 5′ flanking region of the IB exon (IB G1 and IB G2), as well as probes containing the palindromic GATA sites from the IE promoter (IE GG) and GATA-1 intron (GI GG), were bound by both GATA-1 (Fig. 4*A–C*, supershifted band denoted by ^^) and GATA-2 (Fig. 4*A–C*, supershifted band denoted by ^) expressed in COS. We found that the GATA-2 supershifting Ab showed cross-reactivity with GATA-1, as noted by the less intense COS-transfected GATA-1/GATA probe complex recognized by the GATA-2 Ab (Fig. 4*A–C*, probes UE G, IE GG, G1 GG, and IB G2). We also found that the PU.1 Ab recognized a complex of COS-transfected GATA-1 nuclear extracts with several GATA site-containing probes (Fig. 4*A*, 4*B*, probes UE G and G1 GG). The identity of the proteins within this complex is not clear. In our hands, the PU.1 Ab does not recognize GATA-1 protein by either immunoprecipitation or western blot, and no PU.1 protein can be detected by Western blot in the COS-transfected GATA-1 nuclear extracts (Supplemental Data, Supplemental Fig. 2). Possible explanations include the presence of a cross-reacting protein in COS cells that is recognized by the PU.1 Ab; this protein may bind to transfected GATA-1 bound to probe, or the protein may be induced when GATA-1 is expressed.

We then examined DNA binding capacity of GATA factors from mast cell nuclear extracts. In contrast to the nuclear extracts from COS cells, only GATA-2 from mast cells extracts, and not GATA-1, bound putative GATA sites efficiently (Fig. 4*A–C*). We confirmed protein expression of GATA-1 in mast cell extracts as well as transfected COS cell extracts (Fig. 5*C*, Supplemental Data, Supplemental Fig. 2). These findings raise the possibility for tissue-specific post-translational modifications of GATA factors that might affect DNA binding activity and/or the assembly of multiprotein complexes in mast cells necessary for optimal binding to these sequences.

containing a conserved GATA binding site. The UE is a negative control without GATA or PU.1 sites. Whereas GATA-1 and GATA-2 from COS cells bound consensus GATA sites, only GATA-2 from mast cell extracts bound this site. *B*, GATA factor binding to the IE promoter region in vitro. GATA-1 and GATA-2 bind both the IE GG and G1GG probes, which contain the highly conserved GATA palindromic binding sites. Only GATA-2 from mast cell extracts bind these GATA sites. *C*, GATA binding to the exon IB upstream region in vitro. GATA-1 and GATA-2 bind the IB G1 and IB G2 probes which contain conserved GATA binding site. No binding is detected by PU.1 to the IB P1 and IB P2 probes, which contain conserved PU.1 sites. Probe IB is a negative control probe without GATA or PU.1 sites. Only GATA-2 from mast cell extracts bind these GATA sites. *D,* PU.1 binding to the exon IB downstream region. PU.1 binds probes containing GGAA core motifs from the murine sequence (ms IBP3, ms IBP4, ms IBP5, and ms IBP6). *E*, PU.1 also binds to probes with the GGAA core motifs from the human sequence with 5′flanking sequence that is conserved with murine sequence (hu IBP3). Probe hu IB P5 contains the core GGAA sequence, but does not contain the conserved 5′ flanking sequence. Regions of the probes from the IB region are depicted in Fig. 1*D*. Cross-reactivity of the GATA-2 Ab with COS-transfected GATA-1 protein is noted in probes UE G, IE GG, G1 GG and IB G2. A background band is also noted with the PU.1 Ab and COS-transfected GATA-1 extracts with the UE G and G 1 GG probe. BSA, (negative control); G1, GATA-1; G2, GATA-2. *E*, Flanking sequence to core PU.1 motif specify PU.1 binding. The core GGAA motif of the IB probes are aligned and shaded light gray. Probes that bind PU.1 share a 5′ flanking aaga sequence motif and are shaded dark gray. hu IB P5 binds PU.1, but does not share this 5′ flanking motif.

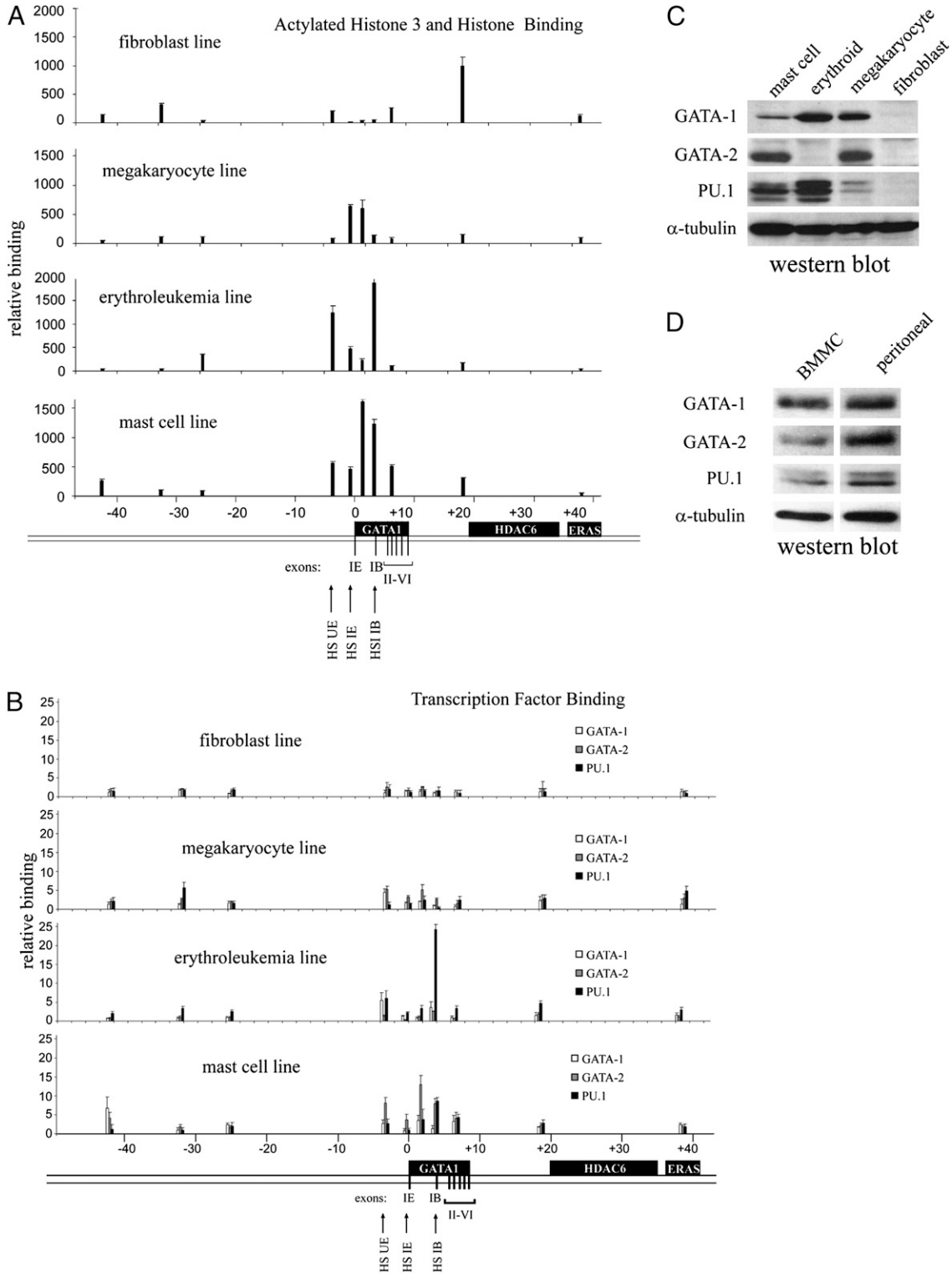

FIGURE 5. GATA-2 and PU.1 bind highly conserved sites of the GATA-1 gene in mast cells in vivo. *A*, ChIP were performed in murine cell lines: fibroblast line (NIH 3T3), megakaryocyte line (L8057), erythroleukemia line (MEL), and mast cell line (C57). Relative binding is measured by the quantitative genomic PCR signal normalized to the no-Ab signal for each PCR primer pair. The *x*-axis is labeled with the relative distances in kilobases from the transcriptional start site of the IE exon. The positions of exons IE, IB, and exons II through VI of the GATA-1 gene are denoted by vertical lines at the bottom of the figure. ChIP for acetylated histones H3 and H4 show areas of open chromatin around the *GATA1* gene in megakaryocyte, erythroid, and mast cell lines. There is relatively little acetylated histone binding in fibroblasts. *B*, In vivo binding of GATA-1, GATA-2, and PU.1 to conserved regions of the GATA-1 gene. GATA-2 binds highly conserved regions in mast cells. PU.1 and GATA-2 co-occupy a highly conserved region in proximity to the IB exon in murine mast cells. *C*, Protein expression of murine GATA-1, GATA-2, and PU.1 in murine cell lines determined by Western blot analysis. α-Tubulin blot shows equivalent loading. GATA-1, GATA-2, and PU.1 protein is coexpressed in mast cell line C57. *D*, Protein expression of murine GATA-1, GATA-2, and PU.1 in primary murine mast cells show coexpression in BMMCs and peritoneal mast cells.

PU.1 binds a GGAA core motif with specific flanking sequences

In the IB region, we examined PU.1 binding to conserved (boxed sequences denoted by * in Fig. 1*D*) and nonconserved sites. Highly conserved consensus PU.1 sites between mouse and human were identified in the 5′ flanking region of exon IB; however, probes containing these sequences (IB P1 and IB P2) were not bound by COS-expressing PU.1 (Fig. 4*C*). The 3′ region of the IB exon was extremely GA-rich with multiple consensus PU.1 sites (GGAA sequences). The IBP3 probe from both mouse and human sequences contained conserved GGAA sites and formed a specific complex (Fig. 4*D*, 4*E*, denoted by $^{++}$) that was supershifted the PU.1 Ab (denoted by $^{+}$). The IBP5 probe also contained a GGAA core sequence that was conserved in mouse and human, but only the mouse sequence bound PU.1 with high affinity (Fig. 4*D*, 4*E*). Notably, the 5′ sequences that flanked the GGAA core was not conserved between human and mouse in this region. Other putative PU.1 sites within the murine sequence that were not conserved in humans (IBP4 and IBP6) were also able to bind COS-transfected PU.1 protein (Fig. 4*D*). However, with nuclear extracts from murine mast cells, we were able to demonstrate binding with only one probe (IBP5). We confirmed that PU.1 binding to probe sequences was dependent on the GGAA core; bound complexes were competed with wild-type probe, but not with a probe with the GGAA core mutated to TCGC (Supplemental Data, Supplemental Fig. 3).

The observation that PU.1 did not bind to all of the predicted consensus PU.1 sites ex vivo suggested specificity imposed by flanking sequences or cooperative recruitment scenarios. We visually inspected the sequences that flanked the core GGAA binding motif. Probes that bound strongly to PU.1 shared an aaga sequence 5′ to the core motif (Fig. 4*F*, shaded in dark gray). No shared consensus was identified in sequences 3′ to the core GGAA binding motif. These results suggest that the aagaGGAA sequence within the GATA-1 locus represented a high-affinity binding site for PU.1. We next tested in vivo binding of GATA factors and PU.1 to the GATA-1 locus *cis*-elements.

PU.1 and GATA-2 bind the GATA-1 locus in vivo in mast cells

To determine the capacity, specificity, and localization of potential PU.1 and GATA factor binding to GATA-1 locus regulatory sequences in mast cells, we performed scanning chromatin immunoprecipitation (37). Immunoprecipitation with Abs to the acetylated histones H3 and H4 to determine open areas of chromatin (46, 47) was first performed. As expected and shown in Fig. 5*A*, acetylated histones bound the genomic regions corresponding to previously identified hypersensitive sites in the GATA-1 locus in primary erythroid cells, the MEL erythroleukemia cell line, and the L8057 megakaryocyte cell line (41). The same regions were open in the murine C57 mast cell line. There is minimal acetylated histone binding detected in the NIH 3T3 fibroblast cell line, in which GATA-1 is not expressed, except in a region in close proximity to the ubiquitously expressed HDAC6 promoter, which had been previously shown (41). Next, Abs for PU.1, GATA-1, and GATA-2 were used to determine in vivo binding of these tissue-specific transcriptional regulatory factors to highly conserved regions in the GATA-1 locus. As shown in Fig. 5*B*, GATA-2 occupies regions surrounding the IE and IB exons in mast cells. PU.1 appears to occupy only the region of the IB exon, but not the IE exon. GATA-1, however, did not bind appreciably to these regions. In MEL cells, GATA-1 was bound within the vicinity of the upstream hypersensitivity (HS UE) region, consistent with the findings reported by Valverde-Garduno et al. (41); in contrast, we did not find appreciable GATA-1 binding to the IE and IB regions in MEL cells. The MEL cells are erythroleukemia cells with high expression of PU.1 (Fig. 5*C*). We also found that PU.1 occupied the GATA-1 IB region in MEL cells. Unlike mast cells, however, the IB region in MEL cells was not co-occupied by GATA-2. We examined the expression of PU.1, GATA-1, and GATA-2 in these cell types. As shown in Fig. 5*C*, the C57 mast cell line expressed all these factors abundantly at the protein level. Primary murine mast cells, (bone marrow derived mast cells and peritoneal mast cells, see Fig. 5*D*) and human mast cells (HMC-1 and skin mast cells) (48) also express these transcription factors. In addition, GATA-1, GATA-2, and PU.1 were readily detected from the murine megakaryocyte line (L8057). The murine erythroleukemia cell line MEL is transformed by activation of PU.1 expression, and thus high levels of PU.1 are detected in addition to GATA-1. A significant amount of GATA-2 protein is not seen in MEL cells, and the fibroblast line does not express these transcription factors (Fig. 5*C*).

PU.1 activates a genomic region flanking the IB GATA-1 exon

To determine the potential functional activity of the genomic regions binding GATA-2 and PU.1 in vivo, we performed reporter assays in the human mast cell line, HMC-1 and the human epithelial cell line, HeLa. Genomic sequences containing the highly conserved regions of the murine GATA-1 genome were placed upstream and downstream of the SV40 promoter-containing reporter, pGL2pro. These genomic fragments included the upstream enhancer (UE) region, the promoter of the IE exon (IEP), the GP2, and the area 5′ of the IB exon (IBa and IBb) and the downstream area 3′ of the exon (IBds; Fig. 6*A*). Although GATA-2 was found to bind the upstream enhancer, the 5′ region of the IB exon, and the GATA palindrome sites both in vivo and ex vivo, neither GATA-2 nor GATA-1 could activate transcription of genomic fragments containing these sites in luciferase assays in HMC-1 (Fig. 6*B*). These murine genomic regions also contained putative PU.1 binding sites (not conserved); similarly, PU.1 did not activate luciferase reporters with these genomic fragments in HMC-1. In contrast, both GATA-1 and GATA-2, as well as PU.1, were able to activate transcription of these genomic fragments in HeLa (Fig. 6*C*). Expression of transfected proteins were confirmed by Western blotting (Supplemental Data, Supplemental Fig. 4)

We found that PU.1 was able to activate transcription from a GA-rich genomic sequence downstream of the IB exon that was homologous between mouse and human (reporter construct IBds; Fig. 6*B*, 6*C*, Supplemental Data, Supplemental Fig. 5). The human genomic region contains two PU.1 binding sites and the murine region contains four PU.1 binding sites that were verified by EMSA. This region was also bound in vivo by PU.1 in murine mast cells. The capacity for PU.1 to transactivate the murine sequence did not depend on the orientation of the sequence, which is consistent with enhancer function. Mutations of single PU.1 sites did not significantly reduce transactivation potential of PU.1. However, mutations of all four PU.1 binding sites abrogated the ability of PU.1 to activate this sequence (Fig. 6*D*). PU.1 activation of the human sequence was also independent of orientation in HeLa. In HMC-1, PU.1 transactivated this human genomic region when placed upstream of the luciferase gene; however, it did not significantly transactivate when placed downstream (Supplemental Data, Supplemental Fig. 5). Cotransfection of the GATA factors with PU.1 into HMC-1 or HeLa cells did not result in synergistic transcriptional activation (data not shown). These findings suggest that regulation by the GATA factors and PU.1 may be cell type-dependent. These data show that conserved *cis*-elements downstream of the IB exon are regulated by PU.1, but the in vivo control of GATA-1 expression in mast cells likely depends on other genomic elements and appropriate chromatin structure that may not be accounted for in these assays.

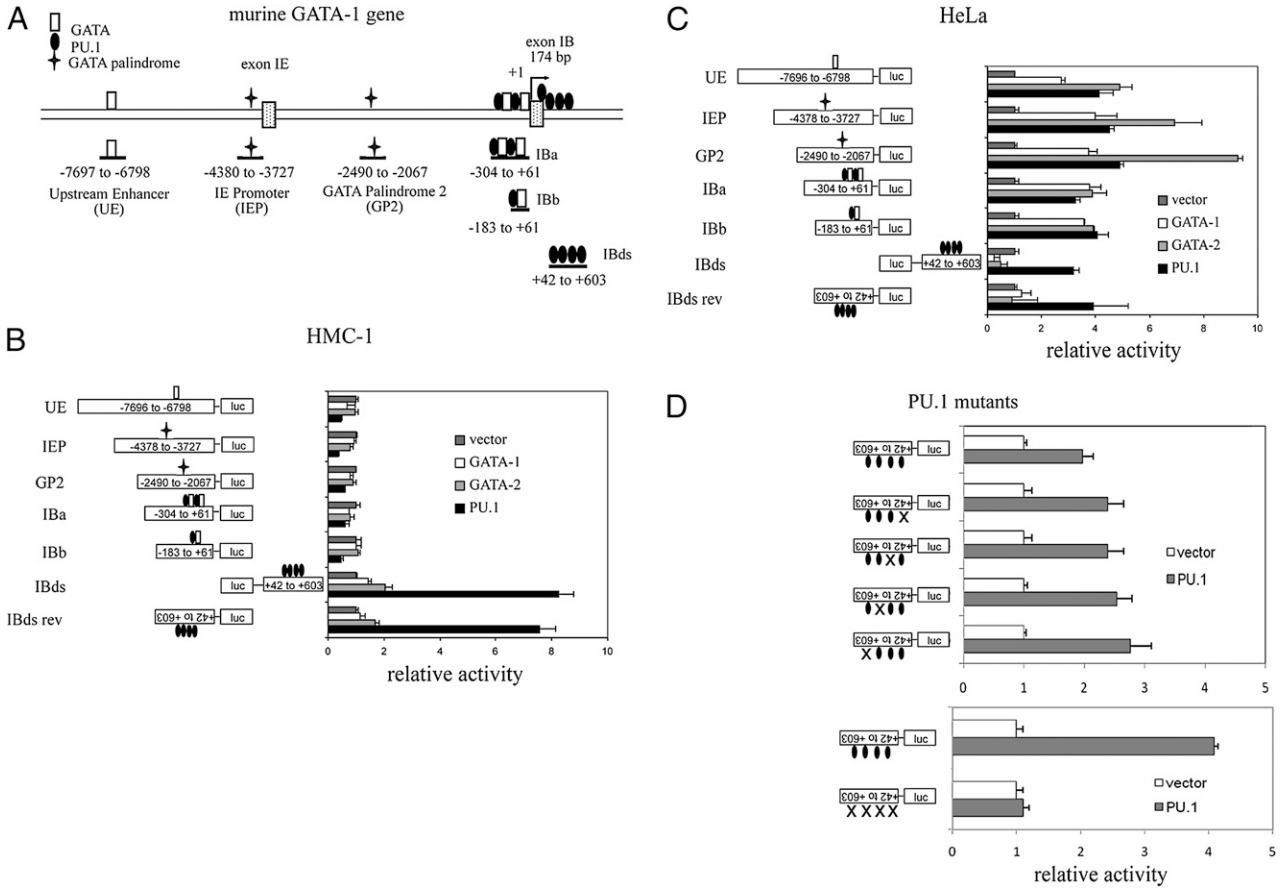

FIGURE 6. PU.1 transactivates a conserved region downstream of the mast cell exon. *A*, Schematic representation of the GATA-1 gene with genomic regions used for reporter assays. Conserved transcription factor binding sites are shown. GATA binding sites are denoted by rectangles; PU.1 binding sites are denoted by black ovals; GATA palindrome sites are denoted by stars. UE, upstream enhancer; IEP, IE promoter; GP2, GATA palindrome within GATA-1 intron; IBds, genomic region downstream of the IB exon. *B*, Reporter assays in the human mast cell line, HMC-1 showing transactivation potential of GATA-1, GATA-2, and PU.1 on the various genomic regions. Genomic fragments were placed in the pGL2pro vector, which has a SV40 promoter upstream of the luciferase gene. Neither GATA-1 nor GATA-2 transactivate conserved regions with GATA sites in HMC-1. PU.1 transactivates a conserved region downstream of the IB exon. The sequence is activated by PU.1 when placed downstream (IBds) or upstream (IBds rev) of the luciferase gene. *C*, Reporter assays in the human epithelial cell line HeLa. In contrast to HMC-1 cells, both GATA-1 and GATA-2 transactivate conserved GATA sites in HeLa. Similar to HMC-1, PU.1 also transactivates the conserved region downstream of the IB exon in HeLa cells in both orientations (IBds and IBds rev). This downstream IB region does not contain GATA sites and is not activated by the GATA factors. The other regions (UE, IEP, GP2, IBa, and IBb) contain GGAA sequences and are activated by PU.1 in HeLa, but not HMC-1. *D*, Reporter constructs with single mutations in each of the four PU.1 binding sites in the region downstream of the IB exon are activated by PU.1 in HeLa cells. However, mutations of all four PU.1 binding sites abrogates the ability of PU.1 to transactivate this reporter. Black ovals denote PU.1 binding sites, and white rectangles denote GATA binding sites. The x denotes mutated PU.1 binding site.

PU.1 is required for the expression of the mast cell isoform of GATA-1

Fetal liver cells derived from PU.1$^{-/-}$ mice can be maintained in culture with IL-3, but they cannot differentiate into mast cells. Restoration of PU.1 expression rescues the capacity for mast cell development (16). We examined the expression of the GATA-1 isoforms in PU.1$^{-/-}$ fetal liver cells by RT-PCR. As shown in Fig. 7*D*, the IE exon containing isoform of GATA-1, but not the IB exon containing isoform, is expressed in PU.1 deficient cells. GATA-2 is also expressed in these cells as previously described (16) (Fig. 7*B*, Supplemental Data, Supplemental Fig. 6). By Western blot, GATA-1 protein is detectable in the PU.1$^{-/-}$ cells, but at a much lower level than in mast cells. Retrovirally expressed PU.1 in the PU.1$^{-/-}$ cells cultured in IL-3 and SCF for 2 wk differentiate into mast cells, by expression of granules morphologically, and by expression of IgE receptor and c-Kit by flow cytometry. Concurrent with differentiation, we found that restoration of PU.1 resulted in expression of the IB isoform of GATA-1 (Fig. 7*D*). Furthermore, total GATA-1 protein also appeared to be upregulated with PU.1 expression (Fig. 7*C*). This finding suggested that PU.1 directly or indirectly targeted the IB isoform of GATA-1 in mast cells, but was not required for IE expression. We have also retrovirally restored expression of GATA-1 into the PU.1$^{-/-}$ fetal liver cells; however, GATA-1 expression alone in the absence of PU.1 does not rescue mast cell differentiation by morphologic or flow cytometric analysis (data not shown). This finding suggests that PU.1 targets other factors in addition to GATA-1 to regulate mast cell differentiation.

Discussion

In this study, we have investigated the regulation of GATA-1 expression in mast cells. We have identified the full length sequence of the variant murine IB GATA-1 isoform, and we found that this isoform is abundantly expressed in mast cells. Our studies suggest that PU.1, and possibly GATA-2, may be the critical regulators of GATA-1 expression in mast cells, based on the demonstration of in vivo and in vitro binding to conserved genomic elements of the GATA-1 gene. Furthermore, reporter assays and in vivo studies

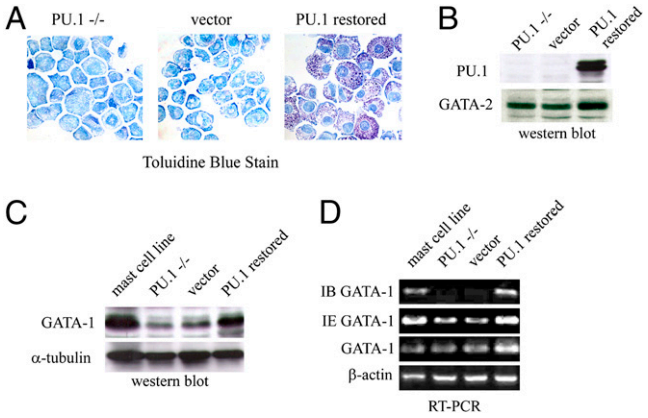

FIGURE 7. PU.1 is required for GATA-IB expression. *A*, PU.1$^{-/-}$ fetal liver cells cannot differentiate into mast cells. Retroviral restoration of PU.1 expression rescues mast cell differentiation. Cytospin preparation of cells stained with toluidine blue. Photomicrograph images were acquired with a Kontron ProgRes 3012 digital camera and Roche Image analysis software (Roche, Tuscon, AZ) with a Zeiss Axiophot microscope (Carl Zeiss, Thornwood, NY). Original magnification ×40. Western blot shows restoration of protein levels of PU.1. *B*, Western blot shows GATA-2 expression in PU.1$^{-/-}$ cells that does not change significantly with reconstitution with PU.1. *C*, Western blot shows upregulation of GATA-1 at the protein level with PU.1 expression. α-Tubulin shows equivalent loading. *D*, PU.1 upregulates IB GATA-1 expression. RT-PCR analysis of GATA-1 isoforms shows expression of IE GATA-1 isoform in undifferentiated cells, but no expression of IB GATA-1. The expression of IB GATA-1 is upregulated with restoration of PU.1 expression. β-actin shows equivalent loading.

with PU.1-deficient cells support the hypothesis that murine IB exon expression is dependent on PU.1. These findings suggest a model in which PU.1 and possibly GATA-2, two transcription factors critical for early mast cell differentiation, cooperate to regulate the expression of a downstream transcriptional regulator, GATA-1, through mast cell regulatory elements. This a mechanism might explain the conundrum of how the antagonistic PU.1 and GATA factors are coexpressed in mast cells. Our studies define a regulatory network linking the GATA factors and PU.1 in mast cells.

Our findings suggest that binding of the GATA factors to the GATA-1 locus is dependent on cell type. Both GATA-1 and GATA-2 bind consensus GATA motifs in vitro. However, in mast cells, only GATA-2 can be demonstrated to occupy these regions both in vitro (with nuclear extracts from mast cells) and in vivo (by ChIP). This observation suggests that mast cell-specific complexes may constrain DNA recognition. The GATA factors activate transcription of luciferase reporters containing conserved GATA sites in a non-mast cell line (HeLa); these sites lie within regions important for GATA-1 expression in erythroid cells. However, GATA factors do not activate reporters containing these conserved sites when transfected in a mast cell line (HMC-1). Although we show in vivo and in vitro binding of GATA-2 to the GATA-1 locus, our data demonstrate the capacity of only PU.1, and not GATA-2, to transactivate the *GATA1* gene. These findings might suggest the presence of mast cell factors that negatively regulate GATA transcriptional activity on the erythroid promoters/enhancers or the requirement of additional enhancers. Another possibility is that the endogenous GATA-2 expression in HMC-1 might blunt enhancement of transactivation by transfected GATA proteins. However, we have found that the basal transcriptional activity of reporters with GATA-site containing *cis*-elements do not differ significantly from empty vector reporters (data not shown). This finding suggests that endogenous GATA-2 expression in HMC-1 cells does not have significant transcriptional activity on the GATA-site containing reporters. Furthermore, because reporter assays might not reflect the influence of chromatin configuration or regulation by long-distance enhancers, additional studies are needed to clarify the role of GATA-2 in the regulation of GATA-1.

Transcription factor binding might also be influenced by conserved sequences that flank core binding motifs. We have demonstrated high-affinity binding of PU.1 in vitro to the core GGAA sequence flanked at its 5′ end by AAGA. These sequences are found within conserved regions of the GATA-1 locus that are occupied in vivo by PU.1 in mast cells. This particular sequence motif has also been validated to be a binding site within promoters and enhancers of a number of confirmed PU.1 target genes in both myeloid and lymphoid cells (49). Additional studies are needed to determine whether this motif regulates PU.1-dependent activation of other critical mast cell genes.

An important negative finding was that forced expression of GATA-1 did not rescue mast cell differentiation in PU.1$^{-/-}$ fetal liver cells. This result suggests that GATA-1 requires the expression of other PU.1 target genes to specify the mast cell lineage. Consistent with this notion is the finding that PU.1 deficient cells are immature and do not express any typical markers of mast cell differentiation (16). In contrast, GATA-1 null cells have the capacity to develop into mature mast cells in culture, whereas GATA-1–deficient mast cells hyperproliferate, but do not terminally differentiate in vivo. We speculate that PU.1 regulates other genes that are critical for the early stages of mast cell development in addition to GATA-1. Another potential reason that GATA-1 does not restore mast cell differentiation in the absence of PU.1 is a possible cooperative interplay between PU.1 and GATA-1. In myeloid and erythroid cells, PU.1 and GATA-1 are antagonistic; PU.1 is dominantly expressed during myeloid development and GATA-1 is upregulated in erythroid differentiation. However, both GATA-1 and PU.1 are required for specification of normal mast cells. Based on this model, forced expression of GATA-1 in the absence of PU.1 would be insufficient to rescue mast cell development. The mechanisms of this potential cooperativity between GATA-1 and PU.1 require further investigation.

We have previously shown that, in fetal liver and yolk sac, the chromatin in both the IE and IB regions is open (4). Other investigators have also identified open chromatin that is sensitive to DNase I close to the IB start site in a multipotential hematopoietic progenitor cell line FDCP-mix (45). In this study, we show that the IE isoform is expressed at low levels in undifferentiated, murine embryonic stem cells, whereas the IB isoform is selectively upregulated during mast cell differentiation. These findings support the notion of a "primed" state of pluripotent cells, in which low levels of GATA-1 and other lineage-specific transcription factors are expressed. During commitment to the erythroid or mast cell lineage, the IE isoform or the IB isoform, respectively, is upregulated. Our studies show that PU.1 is not needed for IE expression, but it is required for IB expression. This finding fits a model in which the myeloid transcription factor PU.1 upregulates the expression of GATA-1 in murine mast cells and eosinophils through the IB exon.

The differential expression of the noncoding IE and IB exons in hematopoietic cell types indicate the presence of cell-specific *cis*-regulatory elements. Given that the IE and IB isoforms are predicted to code for identical proteins, the physiologic importance of this genomic organization may be to permit tissue-specific GATA-1 expression. Although we have not identified a human ortholog of the IB isoform from human mast cell lines, the 5′ flanking sequences of the IB exon is highly conserved between species. We have demonstrated that conserved PU.1 motifs from both mouse

and human sequences in the IB region bind PU.1 in vitro. Furthermore, reporter constructs containing the murine and human conserved *cis*-element can also be transactivated by PU.1 (Supplemental Data, Supplemental Fig. 5). This region has also been found to be DNase 1 hypersensitive and bound by acetylated histones in both mouse and human hematopoietic cells (41). This evidence supports a conserved role for these sequences in the regulation of GATA-1 expression. Thus, the shared biologic function of this enhancer in humans and mice might be the upregulation of GATA-1 expression in mast cells during differentiation; in human mast cells, this upregulation might occur through a GATA-1 transcript other than the IB isoform.

The upstream enhancer of GATA-1 is critical for proper expression of GATA-1 in erythroid cells and megakaryocytes (7, 27, 28, 44). A targeted deletion of this site in mice (the GATA-1 low mouse mutant) also results in an abnormal mast cell phenotype (17, 32). Intriguingly, proper eosinophil development does not appear to be dependent on this enhancer (30). We have examined the expression of GATA-1 in primary bone marrow-derived cells from the GATA-1 low mouse. Both IE and IB isoforms are detectable in these mast cells, suggesting that the expression of either of these isoforms is not solely dependent on this enhancer (data not shown). The role that the upstream enhancer plays in the regulation of GATA-1 expression during mast cell development is currently under investigation. In this study, we found that GATA-2, and not GATA-1, bind conserved genomic regions of the upstream enhancer in mast cells both in vitro and in vivo. Surprisingly, neither GATA-2 nor GATA-1 activated upstream enhancer sequences containing conserved GATA sites in reporter assays in mast cells, suggesting that proper chromatin configuration is developmentally regulated and/or other transcription factor complexes participate in the expression of the gene in vivo.

One of the critical questions that remain is how GATA-2 might cooperate with PU.1 to regulate critical mast cell targets in vivo. In vivo GATA-2 and PU.1 binding sites are not in close proximity within the GATA-1 locus. The majority of sites occupied by GATA-2 are in conserved regions upstream of the IB exon. The two closest sites bound by GATA-2 and PU.1 are separated by ~250 bp. We cannot, however, rule out the possibility of long-range interactions between these two factors in vivo. Another possible mechanism to explain their shared requirement for development is that either or both of these factors might be altering chromatin configurations that regulate access of other transcription factors to the *GATA1* gene. Further analysis of the consensus binding sites and flanking regions recognized by GATA-2 and PU.1 will provide insight into sequence recognition specificity in mast cells and other factors that might co-occupy the gene that participates in its regulation.

Disclosures

The authors have no financial conflicts of interest.

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