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

Clifford M. Takemoto, Stephanie Brandal, Anil G. Jegga, Youl-Nam Lee, Amir Shahlaee, Ye Ying, Rodney DeKoter and Michael A. McDevitt

*J Immunol* published online 19 March 2010
http://www.jimmunol.org/content/early/2010/03/19/jimmunol.0900927
PU.1 Positively Regulates GATA-1 Expression in Mast Cells

Clifford M. Takemoto,* Stephanie Brandal,* Anil G. Jegga,‡ Youl-Nam Lee,* Amir Shahlaee,§ Ye Ying,§ Rodney DeKoter,¶ and Michael A. McDevitt§

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: 900-900.

Mast cells are central effectors in the pathogenesis of allergic and inflammatory disorders, and they also participate in normal host defense (I-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...
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 μM 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, 3000 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, 50 ng/ml mouse recombinant IL-3, and 30 ng/ml mouse recombinant IL-6. 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 (BMMC) were derived from one-week-old the P2 wild type C57BL/6 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 CM supplemented and 50 ng/ml of mouse recombinant SCF as previously described (35). The HMC-1 mast cell lines and the L80577 mast megakaryocytic cell lines were maintained in RPMI 1640 with 10% FBS and 1% penicillin/streptomycin and glutamine. The HeLa cell line, 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. The CM57 murine mast cell line was maintained in DMEM with 10% FBS and 1% penicillin/streptomycin and glutamine.

Plasmids

The pMMP-IRES-puro retroviral vector has been described previously (34). pMMP-IRES-puro PU.1 was constructed by subcloning the P2 wild-type 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 ClaI site engineered at the 3′ end. The reporter assays were constructed by cloning genomic fragments of the GATA-1 or PU.1 cDNAs into the retroviral 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 TCAGC. The primers used to amplify by PCR the genomic fragments are as follows: pUE luciferase forward 5′-GTC CTT CTT CTC TCC CTC CTG CCA-3′, reverse 5′-GGT GCT GGA CTC ATA TCC CAT-3′; pIEP luciferase forward 5′-CCC CAC CCG CCA CAA GAC AGC CTG-3′, reverse 5′-AGT GGT GAG ACT CAA AGG-3′; pG2 luciferase forward 5′-CTT GGT CCT GCA TTC TCT-3′, reverse 5′-GGC CTG GAG-3′; pG2 luciferase forward 5′-CCA AAC CAA CAC ATG ACT CCT TGT T-3′, reverse 5′-GCT GCC GTA GGC TTC TCT-3′; pG2 luciferase forward 5′-GCC AGA ATG CAG CAC GAC GAG-3′, reverse 5′-GGG CTT GAC TTC TCA CCT TT-3′; pIBds luciferase forward 5′-GCA ATG TGG GGG GAG CAG G-3′, reverse 5′-AGT GGT GAG ACT CAA AGG-3′; pIBb luciferase forward 5′-AGT GGT GAG ACT CAA AGG-3′, reverse 5′-GGC CTG GAG-3′; pIBds luciferase forward 5′-CCT GCT GAC TTA TGC G-3′, reverse 5′-TGG GTT CTC CCT TTG GGG C-3′; and pIBb luciferase forward 5′-TGT GGC TCT GCA TTC ACC TCC TTC CTC-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 7.5 μg FuGENE 6 (Life Technologies) and dium 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 CTT CAT ACC ACC AAG AAG-3'; IB GATA-1 forward 5'-AAA ATC CTA TTA TGG GAG GTG TCA AGG-3'; GATA-1 common forward 5'-ATC AGC ACT GCC CTA CTA CAG AG-3'; GATA-1 reverse 5'-GAG AGA AAG GAC TGG GAA AG-3'; PU.1 forward 5'-AGA GCA TAC CAA CTT CAG ACT G-3'; PU.1 reverse 5'-GTT CGG AGA AAT CCC AGT AGT G-3'; GATA-2 forward 5'-CAA GAA AGG GGC TGA ATT TTT CG-3'; GATA-2 reverse 5'-GTT GCC TCC ACC ACG TGC CAT G-3'; β actin forward 5'-GTT ACG AGG CCC AGA GCA AGA G-3'; β actin reverse 5'-GCT CAC AAG AGA TCC ACC TGC-3'. Conventional PCR primers and conditions for IE GATA-1, IB GATA-1, PU.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.betw. the mRNA of GATA-1 and β-actin signal in naive cells and in 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 actin normalized GATA-1 IE or IB signals to the actin normalized GATA-1common 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: −40 bp of the IE GATA-1, forward 5'-CAT CTA CAA CTT CAA CAA A-3'; reverse 5'-ACC ACA TCCA ACA CTA GGA AAA G-3'; −32.5 forward 5'-GCC TGG CCC ACC ATG CCT CA-3'; −32.5 reverse 5'-GTC GAA AAA GAT GGA GGA GG-3'; β actin forward 5'-GTC ACG AGG CCC AGA GCA AGA G-3'; β actin reverse 5'-GCT CAC AAG AGA TCC ACC TGC-3'. Conventional PCR primers and conditions for IE GATA-1, IB GATA-1, GATA-2, PU.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.betw. the signal of GATA-1 IE or IB signals, the ratio of the actin normalized GATA-1IE or IB signals to the β-actin normalized GATA-1common signal was determined.

**Results**

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 × 105 cells were placed in 1500 μl RPMI 1640 with 10% FBS in 24-well plates. A total of 0.5 μg of DNA was used at DNA-to-reagent ratio of 1:2.5. The driver-to-reporter ratio was 1:4. Mouse pansey 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 × 105 cells were placed in 100 μl DMEM with 10% FBS in 24-well plates. A total of 0.5 μg of DNA was used at DNA-to-reagent ratio of 1:24. The driver-to-reporter ratio was 1:4. Mouse pansey 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 the level of internal control.

**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 S’RACE in a well-characterized murine mast cell line C57. Two abundant RACE products were found.
amplified and sequenced: a ∼600-bp product and a larger ∼700-bp product (Fig. 1A). 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. 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 *.

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.
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. 1C, 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. 1B–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. 2A, 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. 2A). 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. 2B, 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. 2B).

![FIGURE 2.](image)

The Journal of Immunology

![FIGURE 3.](image)
FIGURE 4. EMSAs demonstrate GATA-2 and PU.1 binding to consensus binding sites. Regions of EMSA probes are denoted in Figs. 1D 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 [32P]-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 identify 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 (http://www.gene-regulation.com/index.html) to inspect ~110,000 bp of human and murine GATA-1 sequences which 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 IE 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. 4A–C, supershifted band denoted by ^^) and GATA-2 (Fig. 4A–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. 4A–C, probes UE G, IE GG, GI G1, 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. 4A, 4B, probes UE G and GI 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 cell extracts, and not GATA-1, bound putative GATA sites efficiently (Fig. 4A–C). We confirmed protein expression of GATA-1 in mast cell extracts as well as transfected COS cell extracts (Fig. 5C, 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.
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. 1D) 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. 4C). 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. 4D, 4E, 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. 4D, 4E).

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. 4D). 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. 4F, 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. 5A, 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. 5B, 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. 5C). 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. 5C, 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. 5D) 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. 5C).

**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. 6A). Although GATA-2 was found to bind the upstream enhancer, the 5′ region of the IB exon, and the GATA palindromic 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. 6B). 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. 6C). 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. 6B, 6C, 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. 6D). 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.
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. 7D, 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. 7B, 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. 7D). Furthermore, total GATA-1 protein also appeared to be upregulated with PU.1 expression (Fig. 7C). 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
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 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 GGA 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 IE 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 IE isoform from human mast cell lines, the 5′ flanking sequences of the IB isoform is highly conserved between species. We have demonstrated that conserved PU.1 motifs from both mouse and human can interact with the 5′ proximal promoter.
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 I 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 up-regulation 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 IE 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.

References


SUPPLEMENTARY DATA

Figure S1: Phylogenetic sequence comparison analysis to identify conserved transcription factor binding sites. Three genomic regions conserved between the mouse (ms) and human (hu) GATA-1 gene were determined with the TRAFAC software program: 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). Conserved blocks are denoted by colored quadrilateral; percent sequence similarity (percent identical) is shown by the dark line. The number of transcription factor binding sites within conserved regions (hits) is depicted by the lighter line. Putative GATA sites are denoted by green rectangles and PU.1 sites are denoted by blue ovals. The GATA palindromic sites are depicted by purple stars.

Figure S2: Protein expression of GATA-1 and GATA-2 and PU.1 in nuclear extracts from transfected COS cells. Western blot analysis for GATA-1, GATA-2 and PU.1 of nuclear extracts from COS transfected cells demonstrate expression of protein.
**Figure S3: Specificity of PU.1 binding to sequences in the GATA-1 gene.** EMSA with the probes containing the GGAA binding motifs from the mouse GATA-1 gene (ms IBP3, ms IBP4, ms IBP5 and ms IBP6) and the human GATA-1 gene (hu IBP3 and hu IBP5). Nuclear extracts (NE) for COS cells transfected with PU.1 (p) show DNA:protein complexes (denoted by ^\^) with ms IBP3, ms IBP4, ms IBP5, ms IBP6 and hu IBP3. Vector transfected COS cells nuclear extracts (v) do not show these complexes. These complexes can be supershifted with a PU.1 specific antibody (denoted by ^). Unlabeled wild type probe (wt) competes for binding of the radiolabeled probe, while unlabeled probe with the GGAA core mutated (mt) does not compete for binding. * denotes background DNA:protein complex and ** denotes free probe.
**Figure S4: Overexpression of transfected transcription factors in HMC-1 and HeLa cells.** The GATA-1, GATA-2 and PU.1 expression constructs contain a FLAG epitope. An antibody to the FLAG epitope was used for western blotting and demonstrates expression of the transfected protein in both HMC-1 cells and HeLa cells. Antibodies specific for GATA-1, GATA-2 and PU.1 were also used to detect endogenous and overexpressed protein. HeLa does not express appreciable amounts of endogenous GATA-1, GATA-2 and PU.1. HMC-1 expresses endogenous GATA-2, but relative little GATA-1 and PU.1. α-tubulin blot demonstrates equivalent loading between samples.
Figure S5: PU.1 activates the conserved human cis-element that is homologous to the murine IB region. The human genomic fragment (grey box) homologous to the murine IB region (red box) contains two conserved PU.1 binding sites (one site is bound with high affinity by EMSA, see figure 4E and S3). The murine region contains four sites that bind PU.1 by EMSA (blue ovals). There is also a GATA binding site in the human sequence (green rectangle) that is not present in the murine sequence. The pGL2 SV40 luciferase reporter construct (hu IBds) contains this human genomic region and is activated by PU.1 in HeLa cells when placed upstream or downstream of luciferase. In HMC-1, this element is significantly activated by PU.1 when placed upstream of the luciferase gene. The human genomic element is also transactivated by GATA-1 and GATA-2 in HeLa cells and by GATA-2 in HMC-1 mast cells.
Figure S6: Expression of GATA-1 and GATA-2 in PU.1 -/- cells and mast cells. RT-PCR analysis show expression of the GATA factors from the C57 mast cell line and PU.1 -/- fetal liver cells. PU.1 is detected only from the mast cell line.