GATA Motifs Regulate Early Hematopoietic Lineage-Specific Expression of the Gata2 Gene

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Transcription factor GATA-2 is essential for definitive hematopoiesis, which developmentally emerges from the para-aortic splanchnopleura (P-Sp). The expression of a green fluorescent protein (GFP) reporter placed under the control of a 3.1-kbp Gata2 gene regulatory domain (2) to the distal first exon (IS) mirrored that of the endogenous Gata2 gene within the P-Sp and yolk sac (YS) blood islands of embryonic day (E) 9.5 murine embryos. The P-Sp- and YS-derived GFP+ fraction of flow-sorted cells dissociated from E9.5 transgenic embryos contained far more CD34+/c-Kit+ cells than the GFP− fraction did. When cultured in vitro, the P-Sp GFP+ cells generated both immature hematopoietic and endothelial cell clusters. Detailed transgenic mouse reporter expression analyses demonstrate that five GATA motifs within the 3.1-kbp Gata2 early hematopoietic regulatory domain (G2-EHRD) were essential for GFP expression within the dorsal aortic wall, where hemangioblasts, the earliest precursors possessing both hematopoietic and vascular developmental potential, are thought to reside. These results thus show that the Gata2 gene IS promoter is regulated by a GATA factor(s) and selectively marks putative hematopoietic/endothelial precursor cells within the P-Sp.

The developmental origin of definitive hematopoietic precursors within a conceptus has been the subject of considerable study and debate. Several lines of evidence suggest that definitive hematopoietic progenitors originate from the para-aortic splanchnopleura (P-Sp) within the embryo proper (9, 26, 33). Lending credence to this hypothesis, Cunato et al. (5) reported that the caudal intraembryonic splanchnopleura of E7.5 to 8.5 murine embryos could give rise to mixed lymphoid-myeloid colonies in vitro. Similarly, human P-Sp cells produced mixed lymphohematopoietic colonies after culture on murine stromal cells or in fetal thymic organ culture. In contrast, human yolk sac (YS) cells did not produce lymphocytes in culture (55). Furthermore, P-Sp-derived but not YS-derived cells from ~E8.5 embryos generated hematopoietic cells capable of long-term reconstitution in sublethally irradiated Rag2−/− mice (6). The developmental importance of the embryonic mesoderm in hematopoietic ontogeny has also been demonstrated in the chicken and frog (8, 59, 60).

It has been alternatively postulated that definitive hematopoietic stem cells migrate en route from the yolk sac (15, 42, 64) to the fetal liver and eventually populate the adult hematopoietic compartment. However, hematopoietic precursors isolated from E7.5 to 8.5 yolk sacs were reportedly incapable of producing lymphoid-myeloid colonies in vitro (5). While one group had reported successful hematopoietic repopulation using E8.5 YS cells in sublethally irradiated mice (25), these cells were generally defective in reconstituting the hematopoietic panoply of adult mice exposed to sublethal irradiation (6, 27, 33).

The vertebrate GATA transcription factor family comprises six members (reviewed in references 20, 39, 40, and 63) and each is expressed in a tissue- and developmental stage-specific manner. Of the six GATA factors, GATA-1, GATA-2, and GATA-3, contribute to different aspects of hematopoietic development and are categorized as the “hematopoietic” GATA factors (39), although GATA-2 and GATA-3 additionally contribute to numerous aspects of organ and tissue development outside of hematopoiesis. GATA-1 is necessary for the maturation of primitive and definitive erythroid cells (49, 51), megakaryocytes (61), eosinophils (13), and mast cells (12), whereas GATA-3 plays an indispensable role in the development of T cells (56, 65). In contrast, GATA-2 is indispensable for all hematopoiesis, since gene targeted loss of Gata2 in the mouse germ line leads to embryonic lethality as a consequence of the failure to expand the progenitor pool (58).

Consistent with this conclusion, analysis of chimeric embryos generated using marked Gata2−/− embryonic stem (ES) cells indicated that these cells failed to contribute to any hematopoietic lineage. Several lines of evidence suggest that GATA-2 is expressed in pluripotent hematopoietic stem cells (41) and possibly in hemangioblasts, believed to represent the earliest common precursor of the hematopoietic and endothelial lineages (2, 3, 6, 40). Gata2 haploinsufficiency reduced the number of early hematopoietic stem cells and impaired the quality of both embryonic and adult hematopoietic stem cells, demonstrating that GATA-2 achieves its normal function only with a diploid contribution of factor (22). Interestingly, GATA-2...
appears to be dispensable for the development of endothelial cells, as no vascular defects are evident in Gata2-deficient embryos (58). However, the precise contribution of GATA-2 to the earliest events that define hematopoiesis remains unclear.

We previously showed that the mouse Gata2 gene has two first exons (28). The gene-distal first exon (IS) is specifically expressed in hematopoietic and neural cells, whereas the gene-proximal first exon (IG) is transcribed in almost all Gata2-expressing cells (29). Differential utilization of two distinct first exons has also been demonstrated for the human and chicken Gata2 genes (37, 44). We also reported that the murine Gata2 gene has two regulatory exons has also been demonstrated for the human and chicken Gata2 genes (37, 44). We also reported that the murine Gata2 gene has two regulatory exons has also been demonstrated for the human and chicken Gata2 genes (37, 44). We also reported that the murine Gata2 gene has two regulatory exons has also been demonstrated for the human and chicken Gata2 genes (37, 44). We also reported that the murine Gata2 gene has two regulatory exons has also been demonstrated for the human and chicken Gata2 genes (37, 44). We also reported that the murine Gata2 gene has two regulatory exons has also been demonstrated for the human and chicken Gata2 genes (37, 44). We also reported that the murine Gata2 gene has two regulatory exons has also been demonstrated for the human and chicken Gata2 genes (37, 44). We also reported that the murine Gata2 gene has two regulatory exons has also been demonstrated for the human and chicken Gata2 genes (37, 44).

In this study, we investigated four aspects of GATA-2 regulation in early hematopoietic cells. First, we identified a 3.1-kbp domain 5' to the IS exon that recapitulates Gata2 gene expression in the P-Sp and YS and named this region the Gata2 gene early hematopoietic regulatory domain (G2-EHRD). Second, we determined that G2-EHRD-directed green fluorescent protein (GFP) reporter transgenes accurately reflected the endogenous Gata2 expression pattern. When we performed detailed characterization of the GFP-marked cells in the P-Sp and yolk sac, we found that the P-Sp-derived and G2-EHRD-directed GFP+ cells could differentiate into hematopoietic as well as endothelial cells when cultured on OP9 stromal cells. Finally, we elucidated a set of indispensable GATA motifs within G2-EHRD, clarifying in vivo a key Gata2 cis-regulatory element that is active in early embryonic hematopoietic tissues. Thus, this study defines cis elements that contribute to the regulatory mechanisms underlying Gata2 expression in progenitor cells that appear to be able to adopt either a hematopoietic or endothelial developmental fate.

### MATERIALS AND METHODS

**Construction of transgenes.** The mouse Gata2 expression construct p7.0ISGFP (29) was used as the base vector. Genomic DNA was recovered from a 129/SV mouse genomic library (Stratagene). The delF1 construct was prepared by ligating the following DNA fragments together, in order: a PCR product including 412 bp 5' to the SfiI site, the SfiI-NotI fragment, including the IS exon of the mouse Gata2 gene, a splice donor-acceptor sequence from pSVβ (Clontech), the GFP gene, and the poly(A) addition site from pSVβ. The restriction enzyme sites for BssHII, BsrGI, SfiI, and XbaI defined the 5'-end of the constructs BshHII-BS, BsrGI-SF, SfiI-IS, and XbaI-IS, respectively. 3′d26 and 3′d26SIGII were prepared similarly to delF1, but after deletion (by PCR mutagenesis) of the 26-bp GATA/GATG sequence between bp -3036 and -3011 of the promoter. The 5′-IS and 5′-ISH constructs both bear the 336-bp region from the AIIIIS site (3.1 kbp 5′ to the transcription start site) to the SmaI site, as well as all the same fragments included in the other constructs (above).

The authenticity of all constructs was verified by sequencing.

**Transgenic mice assay and screening of transgene-positive mice.** Expression constructs were digested with the appropriate restriction enzymes and removed from vector sequences. DNA was purified by agarose gel electrophoresis and SUPREC columns (Takara) prior to oocyte microinjection. DNA (5 ng/µl) was injected into fertilized mouse eggs using standard protocols (14). For screening of embryos or pups for transgenes, genomic DNA was extracted from the caudal part of E9.5 embryos or a 2-mm length of newborn mouse tails with 50 mM sodium hydroxide at 95°C for 30 min. The DNA solution was neutralized by 1 M Tris-HCl (pH 8.0). PCR was performed with the Gene Amp PCR system 9700 (Perkin-Elmer). Primer sets for genotyping using the GFP gene are shown in Table 1.

**Gene line targeting and genotyping.** The Gata2+/- line was the generous gift of Stu Orkin (58). Gata2 genotypes were determined by genomic PCR using a neo primer set (52) and the wild-type allele primer set described by Tsai et al. (58). The Gata2-GFP knock-in (Gata2-KI) line was generated by inserting GFP coding sequences at the translational start site of the Gata2 locus. The neomycin cassette was eliminated using Cre/loxP-mediated homologous recombination. Detailed analysis of the Gata2-KI mice will be reported elsewhere (unpublished data). The Gata3 mutant mouse line was described as generated (45). Genotyp-
Gata3 was performed by genomic PCR to detect the neomycin cassette and Gata3 wild-type alleles as described by Lim et al. (21).

**Immunohistochemistry.** Wild-type BDF-1 female mice were mated with male D3.1GFP transgenic mice. Noon of the day a vaginal plug was observed was denoted E0.5. D3.1GFP E9.5 embryos were fixed with 4% paraformaldehyde in phosphate-buffered saline for 15 min at 4°C. They were treated with 1% hydrogen peroxide to block endogenous peroxidase activity. Prior to antibody staining, the samples were blocked with 1% goat serum and 0.2% bovine serum albumin in PBS-MT (2% skim milk and 0.1% Triton X-100 in phosphate-buffered saline). RC1.1 rat anti-chicken GATA-2 monoclonal antibody (66) or anti-GFP antibody (Living Colors, Clontech) was added and incubated overnight at 4°C. The samples were then incubated with hors eradish peroxidase-conjugated goat anti-rat secondary antibody for 1 h. The colorimetric substrate detection used was 0.03% diamobenzidine, 0.08% nickel chloride, and 0.15% hydrogen peroxide. The samples were dehydrated and embedded in wax. The whole-mounted samples were also thin sectioned (5 μm). Nuclear fast red was used for counterstaining.

**Flow cytometry.** P-Sp and YS from E9.5 embryos were dissected and treated with Dispase II (Roche Diagnostics) at 37°C for 15 min and then pipetted gently to dissociate the cell clumps. Single cells were stained using the following antibodies (flow cytometry grade; Life Technologies): set 1 (the hematopoietic cell lineage) includes CD34-biotin, streptavidin-RED613 and c-Kit-phycoerythrin; set 2 (the endothelial cell lineage) includes TER119-biotin, Mac-1-biotin, Gr-1-biotin, B220-biotin, and streptavidin-allophycocyanin. Biotin-conjugated antibodies were added first, and after washing, other fluorescent antibodies were added. Samples were then incubated with horseradish peroxidase-conjugated goat anti-rat secondary antibody. The colorimetric substrate detection used was 0.03% diamobenzidine, 0.08% nickel chloride, and 0.15% hydrogen peroxide. The samples were dehydrated and embedded in wax. The whole-mounted samples were also thin sectioned (5 μm). Nuclear fast red was used for counterstaining.

**Semiquantitative reverse transcription-PCR analysis.** GFP+ and GFP− cells were sorted using a FACS Vantage. Total RNA was isolated from sorted cells with ISOGEN-LS (Nippon Gene) and cDNA was reverse transcribed with SensiScript reverse transcriptase (QIAGEN). The amount of cDNA used in PCRs was adjusted by dilution to produce equal amounts of hypoxanthine phosphoribosyltransferase amplicon.

The primers used in this study are listed in Table 1. PCR conditions were varied according to the genes examined, and three different conditions of PCR were employed for semiquantitative PCR. The denaturation temperature was 96°C for all primers, and annealing temperatures for specific primer pairs are listed in Table 1. For the step-down PCR method, 25 cycles with decreasing annealing temperature (0.1°C per cycle) were followed by adding 0, 5, and 10 cycles with the annealing temperature of the 25th cycle, making the total cycle number 25, 30, or 35, respectively.

**Coculture with OP9 stromal cells.** GFP+ and GFP− cells (500 cells/well) were seeded on OP9 layers and cultured for 7 days, the cells were fixed with 4% paraformaldehyde and stained with anti-GFP antibody (Molecular Probes). After incubation for 1 h, the samples were then incubated with horseradish peroxidase-conjugated goat anti-rat secondary antibody. The colorimetric substrate detection used was 0.03% diamobenzidine, 0.08% nickel chloride, and 0.15% hydrogen peroxide. The samples were dehydrated and embedded in wax. The whole-mounted samples were also thin sectioned (5 μm). Nuclear fast red was used for counterstaining.

**Electrophoretic mobility shift assay.** Nuclear extracts were prepared from 293T cells transfected with murine GATA-1, GATA-2, and GATA-3 expression plasmids. All three GATA factor cDNAs were inserted into the vector pBlueScript (Stratagene), and recombinant mouse interleukin-7 (20 ng/ml; Research Diagnostics) were added to the medium. After 8 days of coculture at 37°C, hematopoietic cell numbers were calculated against the total number of cells. Hematopoietic cells were analyzed by fluorescence-activated cell sorting.

**RESULTS**

**Gata2 IS promoter-proximal sequences regulate GFP expression in early hematopoietic cells.** The mouse Gata2 gene contains two promoters/first exons, IS and IG, that are differentially utilized in different cell types (Fig. 1A). We previously showed that 7-kbp sequences flanking the IS promoter (in a transgene called 7.0ISGFP) could appropriately specify Gata2 gene expression in hematopoietic and neural tissues (29). In this study, we prepared a series of 5′ deletions of the 7.0ISGFP expression plasmid and tested each deletion derivative using founder (F0) and stable transgenic analyses. We found that the smaller D3.1GFP transgene (Fig. 1A) could reproduce the expression pattern seen in E9.5 7.0ISGFP transgenic embryos.

Two D3.1GFP transgenic lines were generated (lines 95 and N3), and we found that the developmental expression profiles of GFP reporter were very similar in these two lines. We therefore focused on line 95 for detailed analysis.

To characterize expression of the D3.1GFP transgene in the
GFP mark GFP expression exclusively in cells expressing endogenous/Endogenous c-Kit, CD34, and CD45 (Fig. 2B), and these mRNAs were expressed uniformly in each of the fractions (Fig. 2B).

Since similar cell populations in intra- and extracellular vessels appeared to express both GFP and GATA-2, these results suggested that regulatory elements within the 3.1-kbp Gata2 IS extended promoter could recapitulate Gata2 expression in early hematopoietic tissues. We therefore named this 3.1-kbp region the Gata2 gene early hematopoietic regulatory domain (G2-EHRD). As the dorsal aortic wall has been previously identified as the site of developing hematopoietic precursors (17), the present data further suggest that the GFP+ cells may represent hemogenic endothelial cells (36) or hemangioblasts (4), which are believed to represent the earliest common precursors of the hematopoietic and endothelial lineages.

GFP+ cells from D3.1GFP transgenic embryos contain hematopoietic and endothelial precursors. We next characterized the GFP+ cells recovered from the early hematopoietic tissues of E9.5 D3.1GFP transgenic embryos, as outlined in Fig. 2A, using fluorescence-activated cell sorting. The isolated P-Sp and YS (shown in green) were dissociated into single cells and then sorted by fluorescence-activated cell sorting. Both GFP+ and GFP- fractions were further subjected to gene expression profile, cytological, and in vitro lineage differentiation analyses.

We first analyzed the cells for the expression of several hematopoietic and endothelial lineage genes by semiquantitative reverse transcriptase-coupled PCR (Fig. 2B). As anticipated, the GFP amplicon was found exclusively in the GFP+ fraction except for traces in the P-Sp-GFP- fraction (Fig. 2B); this might be explained by slight contamination of GFP+ cells in the GFP- fraction during flow sorting. Of the three hematopoietic GATA factors, only GATA-2 was expressed specifically in the GFP+ fraction isolated from the P-Sp and yolk sac. This further illustrates that the 3.1-kbp G2-EHRD is able to mark GFP expression exclusively in cells expressing endogenous GATA-2. In contrast, GATA-1 was expressed in both GFP+ and GFP- fractions of the yolk sac, while GATA-3 was expressed uniformly in each of the fractions (Fig. 2B).

The hematopoietic markers examined here included SCL, e-Kit, CD34, and CD45 (Fig. 2B), and these mRNAs were significantly more abundant in the GFP+ fraction than in the GFP- fraction. The YS-GFP+ fraction expressed SCL and e-Kit at a higher level than the P-Sp-GFP+ fraction. All endothelial cell markers examined, including CD31, vascular endothelial growth factor receptor 1 (Flt-1), vascular endothelial growth factor receptor 2 (Flk-1), vascular endothelial cadherin, and Tie-2, were expressed at a higher level in the GFP+ fraction than in the GFP- fraction, regardless of the tissue source. Since early expression markers characteristic of the endothelial and hematopoietic lineages were selectively detected in the GFP+ fraction, this suggested that the GFP+ fraction might contain either bipotential precursors or a mixed population of hematopoietic and endothelial committed precursor cells.

Flow-sorted GFP+ cells from P-Sp and YS (Fig. 3A and C, respectively) were then examined histologically. Both populations contained predominantly immature blast-like cells with a characteristic high nuclear to cytoplasmic ratio. In contrast, the majority of the cells in the GFP- fractions from both P-Sp and YS (Fig. 3B and D, respectively) tended to differentiate, in addition to scarce immature blast-like cells. Some of them had the features of erythroid-committed cells, which were pyknotic before enucleation, and cosinophilic cytoplasm, reflecting hemoglobin production.

In the further analysis of P-Sp and YS cell populations, we found that the percentage of c-Kit+/CD34+ cells (revealing possible hallmarks of hematopoietic progenitors) among the total number of recovered GFP+ cells was 16% in the P-Sp and 25% in the YS (Fig. 3E and F, respectively; left green rectangle surrounded by red rectangle), while the same fractions constituted only 1% GFP- cells from either tissue (Fig. 3E and F; left yellow rectangle surrounded by red rectangle). Thus, the GFP+ population contains many more immature hematopoietic progenitors than does the GFP- population. Similarly, Flk-1+/CD31+ cells, which represent endothelial cell progenitors, constituted 47% of GFP+ P-Sp cells and 55% of GFP+ YS cells (Fig. 3E and F, respectively; right green rectangle surrounded by blue rectangle), while these cell populations represented only 5% of the GFP- cells in the P-Sp and 14% of the GFP- YS cells (Fig. 3E and F, right yellow rectangle surrounded by blue rectangle). These data showed that the GFP+ fraction contains 3- to 25-fold more hematopoietic or endothelial precursors than does the GFP- cell population.

The sum of the hematopoietic plus endothelial progenitors in the GFP+ population did not reach 100%. This may be explained in either of two ways: first, the c-Kit+/CD34+ or Flk-1+/CD31+ subsets might not overlap the entire complement of hematopoietic and/or endothelial cell progenitors, or the GFP+ population may be heterogeneous and could contain cells in addition to hematopoietic and endothelial progenitors.

Ex vivo expansion of P-Sp-derived G2-EHRD-GFP+ cells. To examine the developmental potential of GFP+ cells recovered from the D3.1GFP transgenic embryos, we exploited a primary coculture system based on the use of OP9 stromal cells (34) in addition to the cytokines stem cell factor, erythropoietin, interleukin-6 and/or interleukin-7. Culture conditions employing OP9 stromal feeder cells are known to support the differentiation of hematopoietic progenitors into hematopoietic (11) and endothelial (53) cell lineages. GFP+ cells from either P-Sp (Fig. 4A) or YS (Fig. 4C) proliferated and formed colonies after coculture on OP9 cells. These cell clusters contained rounded hematopoietic cells as evident from cytological examination (Fig. 4E, F, and G). While the hematopoietic cells were more abundant in the YS-derived cultures than in P-Sp-derived cultures, only the P-Sp-derived GFP+ fraction sus-
In coculture assays with OP9 cells, GFP sorting of the dissected tissues recovered as single-cell suspensions. In P-Sp and yolk sac, GFP characterization of GFP-expressing and -nonexpressing cells in the E9.5 (A) Schematic presentation of the strategies employed for the characterization of GFP-expressing and -nonexpressing cells in the E9.5 P-Sp and yolk sac. GFP+ and GFP− fractions were obtained by cell sorting of the dissected tissues recovered as single-cell suspensions. In the coculture assays with OP9 cells, GFP+ cells were evaluated by flow cytometry after 8 days of culture. (B) Reverse transcription-PCR analysis of flow-sorted GFP+ and GFP− fractions isolated from E9.5 P-Sp and YS. The genes examined are listed on the left. Equal amounts of cDNAs, adjusted by dilution to produce comparable hypoxanthine phosphoribosyltransferase amplicons, were used for the PCRs. Three different cycle numbers, which gave unsaturated PCR results, were selected for semiquantification. The results of two different cycle numbers are shown in the figure. Samples marked with an asterisk indicate that a higher number (40 and 45) of cycles of PCR amplification were required.

In order to identify the regulatory core sequences within the 3.1-kbp Gata2 EHRD that are required for activity in early P-Sp-derived cells, we analyzed the expression of a series of D3.1GFP deletion mutants in transgenic F0 (founder) assays (Fig. 6A). The 5′ endpoint of the parental D3.1GFP construct started 3,097 bp downstream of the ATG.
upstream of the IS transcriptional initiation site (site A in Fig. 6A). While 7 of 11 transgenic delF1 mutant embryos expressed GFP in a hematopoietic tissue-specific manner, deletion of an additional 26 bp completely abolished the expression of the reporter gene specifically within the dorsal aorta (0 of 9 for the BssHII IS deletion) (Fig. 6A), while expression at other embryonic sites was unaffected. These results indicate that the 26-bp element located between bp 3036 and 3011 5’ to the IS

FIG. 3. Characterization of flow-sorted GFP+ cells derived from E9.5 P-Sp and YS. (A to D) Wright-Giemsa-stained cytospins of GFP+ (A and C) and GFP− (B and D) cells harvested from P-Sp and YS (A and B and C and D, respectively). (E and F) Flow cytometric analysis of P-Sp (E) and YS (F) cells with hematopoietic and endothelial cell surface markers. The left upper panel shows the initial gating. In the middle upper panel, cells were distributed by the intensities of the GFP and side scatter, and GFP+ (green rectangles) and GFP− (yellow rectangles) cell fractions were determined. These fractions were subsequently analyzed with c-Kit and CD34 (hematopoietic cell surface markers, red rectangles) or Flk-1 and CD31 (endothelial cell surface markers, blue rectangles) (lower panels). FSC, forward scatter; SSC, side scatter; CD34-RED613, anti-CD34 antibody labeled with biotin, secondarily labeled by streptavidin conjugated to RED613; c-Kit-APC, anti-c-Kit antibody conjugated with allophycocyanin; CD31-RED613, anti-CD31 antibody labeled with biotin, which was colored by streptavidin conjugated with RED613; Flk-1-PE, anti-Flk-1 antibody labeled with phycoerythrin.
exon is necessary for expression of the GFP reporter gene in early hematopoietic cells.

To further assess the significance of this 26-bp element more rigorously, we deleted it from the parental D3.1GFP construct (3.1d26) as well as from the expression construct, 3.1ISIGII. The latter contains an 8.9-kbp genomic fragment extending from a 5′/H11032 XhoI site to the translation initiation site in the second exon and thus contains both the IS and IG promoters/exons (Fig. 6B). As expected, the mutant 3.1d26 reporter transgene failed to express GFP in early hematopoietic cells (0 of 12). Similarly, while embryos bearing a wild-type 3.1ISIGII transgene exhibited GFP bioluminescence in early definitive hematopoietic tissues (30), the same deletion abolished reporter gene expression in hematopoietic tissues of E9.5 embryos. Thus, we conclude that the 26-bp element is indispensable for regulating Gata2 gene expression in early hematopoietic cells.

We further hypothesized that the 336-bp region, which we designated 5H (located at bp 3097 to 2762 5′ to the IS exon), could contribute to GFP reporter expression (Fig. 6C), since it includes the 2-bp element, it lies near a DNase I-hypersensitive site (29), and it is highly homologous between the human and mouse Gata2 genes. Indeed, upon comparison of the human and mouse sequences, we found that the 5H region en block shows 88% (296 of 336 bp) identity. This inspection also revealed that the region between bp 3036 (delF1 start site) and 2769 (SmaI site) shows 96% (257 of 267 bp) DNA sequence identity.

To ascertain if the 5H region is also sufficient to direct hematopoietic tissue-specific expression of a GFP reporter gene, we prepared two additional expression plasmids in which the 5H region was cis-linked to either an 822-bp Gata2 IS basal promoter (5H-IS) or the heat shock gene protein (HSP) promoter (5H-HSP) (Fig. 6C). As anticipated, the 5H-IS transgene was capable of directing GFP expression in the dorsal aorta, omphalomesenteric and umbilical arteries, liver rudiment, and heart tube of E9.5 transgenic embryos (5 of 15; Fig. 6D and E). This expression pattern in early hematopoietic
tissues is reminiscent of that observed in D3.1GFP transgenic embryos (Fig. 7C to E). While a minority of the 5H-HSP embryos showed variable and weak ectopic expression (data not shown), specific GFP expression in the dorsal aorta, umbilical and omphalomesenteric arteries, and yolk sac in 7 of 13 transgenic embryos was largely identical to that of D3.1GFP transgene-positive embryos (Fig. 6F and G). Hence, these data demonstrate that 5H, containing the 26-bp element, is sufficient for the expression of Gata2 in early definitive hematopoietic tissues in E9.5 embryos. Furthermore, the 5H fragment does not require interaction with the Gata2 1S promoter to achieve early hematopoietic tissue-specific expression.

**Five GATA motifs in the 5H region are indispensable for G2-EHRD activity in vivo.** Casual inspection of the 5H region in G2-EHRD highlighted the presence of six GATA factor-binding motifs, two of which reside in the 26-bp indispensable element (Fig. 7A). We refer to these six GATA motifs, in order (distal from the IS promoter), as 1st-GATA (canonical GATA motif TGATAA), GATG (CGATGG), GATApal (palindromic GATA motif, GATAATC) (57), and 4th-GATA, 5th-GATA, and 6th-GATA (three canonical GATA motifs, AGATAA), respectively. To examine how mutation of these six sites might affect Gata2 gene regulation, we disrupted each GATA motif in the D3.1GFP backbone and analyzed the consequence of those mutations in transgenic founder mouse assays (Fig. 7B to P).

Transgenic embryos bearing the D3.1m1st-GATA, D3.1mGATG, D3.1m5th-GATA, or D3.1m6th-GATA constructs displayed a remarkable reduction of GFP fluorescence in the caudal part of the dorsal aorta or P-Sp (Fig. 7, panels E and F, G and H, M and N, and O and P, respectively; arrows). This reduction can easily be seen in comparison with the parental D3.1GFP transgene (Fig. 7C and D; arrows). In contrast, these transgene-positive embryos displayed a comparable level of
reporter gene expression in the umbilical and omphalomesenteric arteries (arrowheads) to that observed in the parental
D3.1 transgenic embryos; expression was also observed in the liver rudiment (asterisk, Fig. 7C to H and M to P).

In contrast, none of 27 embryos bearing the D3.1m4th-GATA transgene displayed GFP activity in any early hematopoietic tissues, including dorsal aorta/P-Sp, YS, and the umbilical and omphalomesenteric arteries (Fig. 7K and L, arrowheads). Four out of 27 embryos harboring the D3.1m4th-GATA transgene showed ectopic GFP expression (Fig. 7L). In contrast to the fully nullifying effect of the 4th-GATA site mutation, mutation of GATApal did not affect the pattern or intensity of GFP (Fig. 7J). Thus, of the six GATA factor-binding motifs, 1st-GATA, GATG, 5th-GATA, and 6th-GATA are necessary for the expression of GFP reporter in P-Sp but not in YS and umbilical and omphalomesenteric arteries, whereas 4th-GATA is indispensable for the expression of GFP reporter in all of these early hematopoietic tissues.

Both GATA-2 and GATA-3 potentiate early Gata2 gene expression. We envisaged that GATA-3 might be a candidate effector of Gata2 early hematopoietic regulation, because both GATA-2 and GATA-3 are expressed in the P-Sp/aorta-gonad-mesonephros, and those GATA-2- and GATA-3-positive cells have long-term repopulating activity (2). Therefore, to address the question of which GATA factors can activate D3.1, we attempted to clarify whether lack of GATA-2 or GATA-3 might affect Gata2 gene expression. To this end, we crossed the Gata2/H11001/H11002 mice with D3.1 transgenic mice and then bred these compound mutants (Gata2/H11002::D3.1, Gata2/H11001::D3.1, etc.) that were then analyzed at E9.5. Gata2/H11002::D3.1 displayed GFP fluorescence of comparable in-
tensity and with a similar expression profile in the P-Sp (Fig. 8C, arrows), YS, and umbilical and omphalomesenteric arteries (arrowheads) to that of \( \text{Gata2}^{+/+} \) embryos (Fig. 8A and B), demonstrating that D3.1 was faithfully transcribed even in the absence of GATA-2.

Similarly, \( \text{Gata3}^{+/+} \) mice were crossed with \( \text{Gata2} \)-GFP knock-in mice \((\text{G2-KI})\) (30) and compound mutant embryos from the backcrosses were analyzed (Fig. 8D to F). \( \text{Gata3}^{-/-} \) embryos and \( \text{Gata3}^{+/+} \) embryos show GFP expression of similar intensity in the aorta-gonad-mesonephros region at E10.5, indicating that the \( \text{Gata2} \) gene can also be expressed in the absence of GATA-3. In summary, these results suggest that either GATA-2 or GATA-3 can sustain the expression of the \( \text{Gata2} \) gene in early murine hematopoietic progenitors and indicate that these two GATA factors may be functionally compensatory or redundant.

Hematopoietic GATA factors bind to both GATA and GATG motifs. We sought to determine whether the hematopoietic GATA factors (GATA-1, GATA-2, and GATA-3) bind to the GATA and GATG motifs in the 5H region by electrophoretic mobility shift assay (EMSA), employing 1st-GATA and GATG motifs as representatives. Wild-type probe and two mutant probes (m1st-GATA and mGATG) were mixed with nuclear extracts of 293T cells after transfection with GATA-1 (Fig. 9A), GATA-2 (Fig. 9B), or GATA-3 (Fig. 9C) expression plasmid.

**Fig. 7.** GATA motifs in the 5H region are essential for the activity of \( \text{G2-EHRD} \). (A) DNA sequence of the 5H region and location of the GATA motifs (red letters, arrows). (B) Summary of transgenic assays examining \( \text{G2-EHRD} \) mutagenesis. The composition and abbreviations for the transgenes are the same as in Fig. 6. 1st-GATA, GATG, GATApal, and 4th- to 6th-GATA were replaced with CTAG, TCGA, CCATGGC, and CATG, respectively. DA, caudal part of the dorsal aorta; UA, umbilical artery; OMA, omphalomesenteric artery; YS, yolk sac. (C to P) Representative bright-field (C, E, G, I, K, M, and O) and fluorescent (D, F, H, J, L, N, and P) micrographs of embryos bearing D3.1, D3.1m1st-GATA, D3.1mGATG, D3.1mGATApal, D3.1m4th-GATA, D3.1m5th-GATA, and D3.1m6th-GATA transgenes. When the GFP intensity of transgenic embryos with mutated constructs was compared to that of D3.1 transgenic mice, three patterns of GFP expression were observed. First, the D3.1m4th-GATA transgene embryo displayed no GFP fluorescence in any early hematopoietic tissues (except ectopic; L). Second, GFP expression in the P-Sp (arrows) is remarkably reduced in D3.1m1st-GATA (F), D3.1mGATG (H), D3.1m5th-GATA (N), and D3.1m6th-GATA (P) despite persistent GFP expression in the YS and umbilical and omphalomesenteric arteries. Third, D3.1mGATApal is comparable in GFP intensity to D3.1 transgene-positive embryos. Asterisks indicate the liver rudiment.

**Fig. 8.** \( \text{Gata2} \) gene expression in \( \text{Gata2} \)- and \( \text{Gata3} \)-null mutant backgrounds. (A to C) The compound mutant mouse \( \text{Gata2}^{+/+} \) was crossed to a \( \text{Gata2}^{+/-} \) mouse and the resulting embryos of various genotypes were analyzed at E9.5. Bright-field (A) and fluorescent (B) microphotographs of \( \text{Gata2}^{+/-} \) are shown beside a fluorescent microphotograph of \( \text{Gata2}^{+/-} \) (C). The dorsal aorta/P-Sp (arrowheads) and umbilical and omphalomesenteric arteries (arrows) exhibit similar intensities of GFP fluorescence in both embryos. (D to F) A \( \text{Gata3}^{-/-} \) mouse was crossed with a \( \text{Gata2} \)-GFP knock-in \((\text{G2-KI})\) mouse and compound mutant embryos were analyzed. \( \text{Gata3}^{-/-} \) embryos and \( \text{Gata3}^{+/+} \) embryos show GFP expression of similar intensity in the aorta-gonad-mesonephros region at E10.5.
Nuclear extracts prepared from 293T cells transfected with expression plasmids for murine GATA-1 (A), GATA-2 (B), or GATA-3 (C) were incubated with radiolabeled wild-type (WT) m1st-GATA (bearing a mutation in the 1st-GATA site) and mGATG (with a mutation in the GATG site) oligonucleotides. (A) GATA-1 protein was added to the wild-type, m1st-GATA, or mGATG radiolabeled probe in the presence of no (lanes 3, 17, and 24) or a 10-fold excess (lanes 4, 18, and 25) or 100-fold excess (lanes 5, 19, and 26) of unlabeled self-competitor oligonucleotides. Addition of a 10-fold (lanes 6, 8, 10, 20, and 27) or 100-fold (lanes 7, 9, 11, 21, and 28) excess of oligonucleotides bearing one or both GATA mutant motifs resulted in weak or no competition. The anti-GATA-1 N6 antibody supershifted the most prominent complex (lanes 12 and 13), while nonspecific immunoglobulin G (lane 14) did not. As controls, radiolabeled probes were incubated with no extract (lanes 1, 15, and 22) or with nuclear extracts from 293T cells transfected with vector alone (lanes 2, 16, and 23). (B and C) In vitro binding of wild-type, m1st-GATA, and mGATG probes to recombinant GATA-2 and GATA-3 proteins. See the legend to panel A for details. Lanes 1 to 11, 12 to 18, and 19 to 25 correspond to lanes 1 to 11, 15 to 21, and 22 to 28 in A, respectively.

Nuclear extracts prepared from 293T cells transfected with GATA-1 gave rise to three EMSA bands (Fig. 9A, lane 3). Of the three, the lowest-mobility product was due to GATA-1 association, as the N6 anti-GATA-1 antibody specifically supershifted it (Fig. 9A, lanes 12 to 13). Competition experiments using a 10- or 100-fold molar excess of unlabeled oligonucleotide revealed specific competition for the binding of the labeled probe (Fig. 9A, lanes 4, 5, 18, 19, 25 and 26; Fig. 9B and C, lanes 4, 5, 15, 16, 22 and 23). An oligonucleotide bearing mutations in both the GATA and GATG motifs (2M) did not
compete for binding (Fig. 9A, lanes 6, 7, 20, 21, 27 and 28; Fig. 9B and C, lanes 6, 7, 17, 18, 24 and 25). Importantly, unlabeled mutant m1st-GATA and mGATG oligonucleotides partially competed for binding to the wild-type sequence (lanes 8 to 11 in each panel), while mutant 2M did not compete. Thus, both the GATG and 1st-GATA motifs appear to contribute to GATA factor binding.

To examine the binding affinity of the GATA factors for the 1st-GATA and GATG motifs, we used a radiolabeled m1st-GATA probe in the presence of excess unlabeled mGATG oligonucleotide, and vice versa. This analysis revealed that the mGATG EMSA band (with an intact 1st-GATA motif) was hardly affected by the addition of excess unlabeled m1st-GATA oligonucleotide. In contrast, competition of the m1st-GATA oligonucleotide (wherein the GATG motif was unaltered) was markedly reduced in the presence of unlabeled mGATG oligonucleotide (data not shown). Thus, the binding of GATA factors to 1st-GATA appears to be more stable than binding to the GATG motif. Thus, the results of the transgenic reporter assays in concert with EMSA demonstrate that mutations of the GATA and/or GATG sequences in the 5H region of G2-EHRD disrupt the binding of the GATA factor(s).

**DISCUSSION**

In this study, we analyzed GATA-2 expression within embryonic hematopoietic tissues and attempted to define some of the underlying regulatory mechanisms controlling this patternning. During embryogenesis, the intraembryonic hematopoietic centers constitute only a miniscule fraction of the whole embryo. We therefore adopted an approach in which the hematopoietic progenitors would be specifically marked by a GFP reporter transgene under the transcriptional control of the Gata2 early hematopoietic regulatory domain (G2-EHRD).

In this study we identified a 3.1-kbp region 5' to the IS exon that could recapitulate Gata2 gene expression in early hematopoietic tissues. There are now many examples of studies in which reporter gene expression directed by the eukaryotic promoter-proximal regions does not recapitulate the endogenous expression profile (7). This observation suggests that specific combinations of regulatory elements in promoters and enhancers as well as additional chromosomal modulatory elements are crucial for recapitulating the bona fide expression profile. In light of this consideration, we previously executed transgenic rescue assays exploiting a large Gata2 genomic DNA fragment borne on a yeast artificial chromosome. The results suggested that, in addition to G2-EHRD, there is at least one other important regulatory element required for full hematopoietic expression of Gata2 in vivo (66). The expression profile of a lacZ reporter gene inserted into the Gata2 locus within this yeast artificial chromosome recapitulated the endogenous expression profile of Gata2 in hematopoietic tissues throughout development. Importantly, the expression of Gata2 from this yeast artificial chromosome transgene rescued Gata2-null mutant embryonic lethality due to hematopoietic deficiency.

Further analyses examining smaller yeast artificial chromosome clones revealed that the critical regulatory domain for this “full” hematopoietic activity reside within a 5-kbp region lying more than 100 kbp 5’ to the IS exon (our unpublished observation). Although it is quite plausible that G2-EHRD may function in collaboration with this distant enhancer as well as with other, yet undefined hematopoietic elements within the Gata-2 locus, these speculations remain to be explicitly tested in complementation analyses.

It is crucial in this type of analysis to verify the precision of overlap in expression between endogenous and reporter genes. Therefore, we compared the expression of the GFP reporter gene with that of the endogenous Gata2 gene in the hematopoietic tissues of E9.5 embryos using anti-GFP and anti-GATA-2 antibodies. The results demonstrated substantial overlap of the GFP reporter and endogenous Gata2 expression profiles within the vascular and hematopoietic lineages (Fig. 1). Reverse transcription-PCR analysis of GFP+ and GFP− cell fractions also showed a very good correlation between GFP and GATA-2 mRNA accumulation (Fig. 2B). These results thus provide compelling evidence that the 3.1-kbp G2-EHRD contains cis-acting regulatory elements that are required for the expression of the Gata2 gene in early hematopoietic tissues.

Several studies have shown that GATA-2 plays an important role in the emergence of the hematopoietic system from an early developmental stage. The loss of the GATA-2 function in mouse embryos compromises both primitive and definitive hematopoiesis (58), and even half of the normal gene dosage of Gata2 reduces the numbers and compromises the quality of the hematopoietic stem cell population in the aorta-gonad-mesonephros region (22, 30). The existence of a hemangioblast, a presumptive common developmental precursor of both the hematopoietic and vascular systems, has been reported (1, 7). The hemangioblasts develop as endothelial cells lining the major arteries of embryos and in the umbilical cord and then produce hematopoietic cells through budding at the emergence of definitive hematopoiesis (17, 35, 36). The results presented here through immunohistochemical analysis showed that Gata2 and the G2-EHRD GFP reporter transgene are both expressed in cells lining the major embryonic arteries and therefore support reports concluding that GATA-2 is expressed in hemangioblasts or early definitive hematopoietic precursors (18, 19).

The GFP+ fraction contains many hematopoietic and endothelial progenitors, and those recovered from the P-Sp have the potential to differentiate into both endothelial and hematopoietic cells in OP9 cocultures. These data suggest that Gata2 is expressed in hemangioblast-like cells and that these cells can differentiate into both lineages, which is consistent with the contention that Gata2 contributes to the early development of the intraembryonic hematopoietic system.

A key question is whether the developmental potential of hematopoietic precursors and the role of GATA-2 differ within these early hematopoietic cells. We found that, although GFP+ fractions from both the P-Sp and YS gave rise to multilineage hematopoietic colonies after coculture on OP9 cells, only P-Sp-derived colonies exhibited sustained GFP expression, while YS-derived colonies did not. Accordingly, we deduced that the functional role of GATA-2 in the P-Sp progenitors differs from that in the YS. While GATA-2 played an important role in the establishment of hematopoietic precursors in both the P-Sp and YS, it is expressed continuously and is therefore likely to contribute to the maintenance of the hematopoietic precursor pool only in the P-Sp.
Two lines of evidence further suggest a fundamental difference in the nature of GATA-2+ cells derived from the P-Sp and YS. First, when we examined endothelial colony formation on OP9 cells in the presence of vascular endothelial growth factor (Fig. 5D), only P-Sp GFP+ cells led to the formation of endothelial colonies, although endothelial cell surface markers (Flk-1 and CD31) were expressed in GFP+ cells from both P-Sp and YS tissues (Fig. 3F). One plausible explanation is that GFP+ precursors in YS may have more restricted developmental potential than those derived from the P-Sp. A second set of observations suggested a difference in the regulation of Gata2 and, further, that the functional roles played by GATA-2 may differ among early hematopoietic tissues.

The transgenic mouse assays employed a series of G2-EHRD deletion mutants, revealing that the 5H region is both necessary and sufficient for the expression of the GFP reporter gene in the early hematopoietic environment. However, more detailed mutational analyses of the GATA motifs within the 5H region revealed a differential contribution of the GATA motifs to reporter gene expression in the P-Sp versus the YS and the umbilical and omphalomesenteric arteries. Of the six GATA motifs, the 1st-GATA, GATG, 5th-GATA, and 6th-GATA motifs are required only for reporter gene expression in the dorsal aorta/P-Sp but not in the YS, umbilical and omphalomesenteric arteries, or liver rudiment. In contrast, 4th-GATA is indispensable for the reporter gene expression in both the P-Sp and YS and the umbilical and omphalomesenteric arteries, but GATApal is dispensable in both types of tissues.

Whereas the 5H region contains multiple GATA motifs that appeared to be differentially regulated in different tissues, the reason for these differences is currently unknown. The fact that cells lining the dorsal aorta are more likely to be the origin of definitive hematopoiesis than yolk sac cells may provide a plausible explanation for the selective regulation of Gata2 gene expression in these two distinct early hematopoietic tissues and, further, different expression and/or functions for GATA-2. The binding of GATA factor(s) might be influenced by the surrounding sequences that include other transcription factor binding sites, and this contextual difference could allow distinction between each hematopoietic site. This study thus provides an avenue not only for differential analysis of the functional contributions of early hematogenic cells to the development of the hematopoietic and endothelial lineages, but to the different and fundamentally distinctive characteristics observed among these primitive and definitive hematopoietic progenitors as well.

Recently, several groups reported the association of GATA-1 and GATA-2 with the Gata2 gene promoter-proximal region (10, 24, 43). These reports suggest that GATA-2 itself binds within this region and positively autoregulates Gata2 expression, whereas GATA-1 disrupts Gata2 expression through binding to the same region, replacing GATA-2. The present findings are consistent with these reports and demonstrate that the expression of Gata2 is regulated by GATA factors, although GATA-1 may not be the factor that binds to the GATA motifs of the 5H region in vivo in the P-Sp or aorta-gonadal-mesonephros. Indeed, Gata2 gene expression was downregulated when GATA-1 was induced during erythroid cell maturation (50), whereas expression was markedly induced when the Gata1 gene was disrupted in murine ES cells (49, 62) or mouse embryos (our unpublished observation). Similarly, in good agreement with these recent reports, the EMSA experiments showed that all three hematopoietic GATA factors can bind to the 1st-GATA and GATG motifs within the 5H region of G2-EHRD. In preliminary kinetic assays, GATA-2 and GATA-3 tend to bind more tightly to the GATA and GATG motifs of G2-EHRD than does GATA-1 (data not shown), suggesting that high-level expression of GATA-1 is necessary for the replacement of GATA-2 bound in this region by GATA-1.

We propose that GATA-3 is an additional candidate effector of these Gata2 regulatory sequences because both GATA-2 and GATA-3 are expressed in the intraembryonic splanchnopleura (19) and those GATA-2- and GATA-3-positive cells have long-term reconstituting activity (2). There are reports that GATA-3 is expressed in the aortic region and mesonephros at E9 to 10 as well as in aortic hematopoietic clusters, mesodermal cells underlying the aorta, and hematopoietic aggregates in the fetal liver at E11 (23). Thus, the expression profiles, the biochemical properties, and the functional characteristics of the hematopoietic GATA factors support the possibility that GATA-2 and/or GATA-3 may regulate G2-EHRD in vivo. However, we cannot exclude the possibility that other GATA binding factors such as visceral GATA factors (GATA-4 to GATA-6), Evi-1 (32, 46), or MEL1 (31) may also exert regulatory influences on G2-EHRD.

In summary, transgenic mouse lines containing a GFP reporter gene placed under the control of G2-EHRD enabled a detailed analysis of Gata2 expression patterns in early hematopoietic tissues. We adopted this selective labeling strategy in contrast to one that might mark all GATA-2-expressing cells because GATA-2 is normally expressed in many different tissues (63), and recovery of GFP-marked cells from this general population could obscure the results of secondary sorting and culture experiments such as those conducted here. Since G2-EHRD accurately recapitulates Gata2 gene expression in early definitive hematopoiesis in vivo, we plan to utilize this vector to target tissue- and development stage-specific expression of various effector molecules in very early hematopoietic precursors and, by doing so, illuminate the functional roles of these effectors in the commitment of developmentally primitive progenitors to the endothelial or hematopoietic program.

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