GATA-4 Incompletely Substitutes for GATA-1 in Promoting Both Primitive and Definitive Erythropoiesis in Vivo*

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Vertebrate GATA transcription factors have been classified into two subgroups; GATA-1, GATA-2, and GATA-3 are expressed in hematopoietic cells, whereas GATA-4, GATA-5, and GATA-6 are expressed in mesoendoderm-derived tissues. We previously discovered that expression of GATA-2 or GATA-3 under the transcriptional control for the Gata1 gene eliminates lethal anemia in Gata1 germ line mutant mice (Gata1.05/Y). Here, we show that the GATA-4 expression by the same regulatory cassette prolongs the life span of Gata1.05/Y embryos from embryonic day 12.5 to 15.5 but fails to abrogate its embryonic lethality. Gata1.05/Y mice bearing the GATA-4 transgene showed impaired maturation of both primitive and definitive erythroid cells and defective erythroid cell expansion in fetal liver. Moreover, the incidence of apoptosis was observed prominently in primitive erythroid cells. In contrast, a GATA-4-GATA-1 chimeric protein prepared by linking the N-terminal region of GATA-4 to the C-terminal region of GATA-1 significantly promoted the differentiation and survival of primitive erythroid cells, although this protein is still insufficient for rescuing Gata1.05/Y embryos from lethal anemia. These data thus show a functional incompatibility between hematopoietic and endodermal GATA factors in vivo and provide evidence indicating specific roles of the C-terminal region of GATA-1 in primitive erythropoiesis.

The GATA family transcription factors play critical roles in determining cell fate, cell proliferation, differentiation, and the survival of hematopoietic cells. Three of the GATA factors, GATA-1, GATA-2, and GATA-3, are expressed in hematopoietic lineages and are referred to as hematopoietic GATA factors. The other three GATA factors, GATA-4, GATA-5, and GATA-6, are referred to as endodermal GATA factors. Individual hematopoietic GATA factors show a unique expression profile in the hematopoietic system (see Ref. 1 and references therein). Namely, GATA-1 is expressed in erythroid cells, megakaryocytes, cells, eosinophils, and mast cells. Although GATA-2 is expressed in similar cell lineages, its expression in erythroid cells is restricted to immature progenitors. GATA-2 is expressed in hematopoietic stem cells and multipotent progenitors (2). GATA-3 is mainly expressed in Th2 lymphoid cells in the hematopoietic lineage.

Gata1-null mice die around embryonic day 11.5 (E11.5) due to defective maturation of primitive erythroid cells (3). A similar phenotype is observed in mice carrying the Gata1 gene knockdown mutation that causes a 95% reduction in GATA-1 mRNA in erythroid cells (4). We named this knockdown allele Gata1.05. Since the Gata1 gene is located on the X chromosome, this phenotype is observed in hemizygotic male embryos bearing the Gata1.05 mutation (Gata1.05/Y). Although GATA-2 expression is observed at extremely high levels in GATA-1-deficient cells (5), it fails to sustain erythroid cell differentiation. On the other hand, we previously discovered that transgenic expression of either GATA-2 or GATA-3 under the control of the Gata1 gene hematopoietic regulatory domain (G1HRD) (6, 7) successfully rescues Gata1.05/Y embryos from the embryonic lethality (8). Thus, if expressed under regulatory influences associated with the Gata1 gene, both GATA-2 and GATA-3 can replace GATA-1 function in erythroid cells during hematopoietic lineage development.

The question arising from this discovery is whether endodermal GATA factors can substitute for GATA-1 activity when forcibly expressed in erythroid cells. Endodermal GATA factors possess conserved zinc fingers and bind to the consensus GATA box sequence, but their expression profiles and biological roles are quite different from those of the hematopoietic GATA factors (see Ref. 9 and references therein). Therefore, based on the best available knowledge, we surmise the following two possibilities. First, if expressed in erythroid cells under

3 The abbreviations used are: En, embryonic day; G1HRD, Gata1 gene hematopoietic regulatory domain; Gata1.05, Gata1 gene knockdown allele expressing GATA-1 mRNA at 5% of the level of the wild type allele; MuP, mouse a-globin promoter; CFU-E, colony forming unit(s)-erythroid; BFU-E, burst forming unit(s)-erythroid; EpoR, erythropoietin receptor; G4G1, GATA-4-GATA-1 chimeric protein; G4G1R, G4G1 transgene-rescued embryos; CF, C-terminal zinc finger of GATA-1; G1R, Gata1.05/Y mice expressing the GATA-1-HA transgene; G4R, GATA-4-rescued embryos(s); FACS, fluorescence-activated cell sorting; CREB, cAMP-response element-binding protein; HA, hemagglutinin.
GIHRD regulation, the endodermal GATA factors may activate GATA-1 target genes and sustain a physiological level of erythropoiesis. Alternatively, only the hematopoietic GATA factors may be able to compensate for GATA-1 deficiency. A recent study of Drosophila GATA factors supports the latter possibility. In Drosophila, the hematopoietic GATA factor Serpent is essential for the formation of the hematopoietic cells (10–12), whereas the endodermal GATA factor Pannier functions during heart development (13). Forced expression of mouse GATA-4 in Drosophila activates cardiogenic gene transcription and increases the production of cardiac cells, whereas neither the hematopoietic GATA factor Serpent nor mouse GATA-1 can serve as a cardiogenic factor (13). This suggests that the cardiogenic role is evolutionarily conserved among the endodermal GATA factors and cannot be replaced by the hematopoietic GATA factors.

In order to test these two possibilities, we generated transgenic mice expressing GATA-4 under the control of GIHRD and examined its ability to rescue Gata1.05/Y embryos from lethal anemia. Unlike GATA-2 and GATA-3, GATA-4 failed to fully rescue the Gata1.05/Y embryos from lethal anemia, yet it prolonged their survival. This result thus unequivocally demonstrates that GATA-4 incompletely replaces the function of GATA-1 in erythroid cells. To further delineate the difference between GATA-1 and GATA-4, we constructed a GATA-4–GATA-1 (G4G1) chimeric protein in which the N-terminal region of GATA-4 is linked to the C-terminal region of GATA-1 and tested its ability in erythroid cells in vivo. This chimeric protein supported the differentiation and survival of primitive erythroid cells almost completely, but the protein was still insufficient to support definitive erythropoiesis in fetal liver. These results demonstrate that the hematopoietic GATA factors possess conserved functional properties that cannot be compensated for entirely by the endodermal GATA factors.

**EXPERIMENTAL PROCEDURES**

**Construction of Plasmids and Generation of Transgenic Mice**—To generate transgenic mice, mouse GATA-4 cDNA (GenBank™ accession number AF179424) (14) was cloned 3′ of the GIHRD genomic fragment (6). The resultant construct was digested with SalI and purified by a QIAquick gel extraction kit (Qiagen). The G4G1 chimeric cDNA fragment was generated by PCR, verified by DNA sequencing, and inserted 3′ of the GIHRD. To examine the transcriptional activity of the G4G1 chimeric protein, pEF-G4G1HA plasmid containing the EF-1α promoter (15) was constructed and co-transfected into QT6 cells with pRBGP3-MAP, a reporter plasmid containing triplicate mouse GATA sites originating from the α-globin promoter (MAP) and the luciferase gene (16). The luciferase assay was carried out using the Dual-Luciferase reporter assay system (Promega) according to the manufacturer’s instructions. Renilla luciferase activity from the expression plasmid pRL-TK was monitored and used to normalize the firefly luciferase activity. GIHRD–GATA-4 transgenic mice and GIHRD–G4G1HA transgenic mice were generated by microinjection of purified DNA into fertilized BDF1 eggs using standard procedures (17). Founders were screened by PCR using genomic DNA prepared from tail. PCR amplification was carried out using the following primers. For GATA-4 transgenic mice, GATA-4 sense (5′-GATTCACACACCAACGGAAG-3′) and GATA-4 antisense (5′-ATGTCCTGAATCGAGATGC3′) primers were used. For G4G1HA transgenic mice, G4G1 sense (5′-ATGCCCTGTTGCCCTCTATCAG-3′) and G4G1 antisense (5′-TTCTCTGCTGAGATTCCATC-3′) primers were used. The transgenic males obtained were mated with Gata1.05/X mice (4), and the genotypes of the resultant pups and embryos were screened by PCR. The primers used for genotyping (Neo and Zfy) were as described previously (4).

**Immunoblot Analysis**—Whole cell lysates were prepared from the fetal livers and hearts of E12.5 embryos. Briefly, fetal livers or hearts were sonicated in 300 μl of 1× SDS sample buffer and subsequently heat-denatured. SDS-PAGE and immunoblotting were performed using anti-GATA-4 (C-20) or anti-HA (Y-11) antibodies. Anti-LaminB (M-20) antibody was used for normalizing the mass of the proteins. The antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

**Histological Analysis**—E8.5 embryos and E12.5 fetal livers were fixed in 4% paraformaldehyde and embedded in wax. Sections were stained with hematoxylin and eosin. For immunohistochemical analysis, serial sections were processed with anti-GATA-4 antibody and anti-ε-globin antibody (kindly provided by Dr. Tadao Atsumi). Diaminobenzidine was used as chromogen, and methyl green was used for counterstaining. To collect embryonic peripheral blood cells, E12.5 embryos were dissected free of decidual tissues. After removal of the placenta, embryos were washed in 2% fetal bovine serum/phosphate-buffered saline three times and bled from severing the head and umbilical vessels. Peripheral blood cells were washed at 1,500 rpm for 5 min at 4°C. Cytospin samples were prepared from ~30,000 cells spun at 300 rpm for 3 min using CYTOSPIN3 (SHANDON). These preparations were processed for Wright-Giemsa staining. Peripheral blood samples of E18.5 embryos were taken from carotid artery and jugular vein. Peripheral blood smears were prepared and stained with Wright-Giemsa staining.

**Count of Total Fetal Liver Cells**—Single-cell suspensions prepared from E12.5 fetal livers were washed by centrifugation at 1,500 rpm for 5 min at 4°C and resuspended in 1 ml of 2% fetal bovine serum/phosphate-buffered saline. The cell number was counted using a hemocytometer.

**Flow Cytometry Analysis**—Single-cell suspensions from livers or peripheral blood cells were prepared from E12.5 and E13.5 embryos as described above. The cells were incubated in 2% fetal bovine serum/phosphate-buffered saline containing Fc block (CD16/CD32; 1:200 dilution) for 15 min on ice and stained with antibodies for 20 min on ice. The antibodies phycoerythrin-conjugated anti–Ter119, allophyocytocin-conjugated anti-c-Kit, biotin-conjugated CD71, and allophyocytocin-conjugated streptavidin were obtained from BD Biosciences. The apoptotic cells were verified using Annexin V–allophyocytocin according to the manufacturer’s instructions. The samples were analyzed using a FACSCalibur (BD Biosciences). The data were analyzed with the CellQuest program. The experiments were performed three times independently and confirmed the reproducibility.
In Vitro Colony Assays—Fetal livers of E12.5 and E13.5 embryos were dispersed into single-cell suspension. For CFU-E assays, 2 × 10^4 cells were plated in 1 ml of methylcellulose medium (MethoCult M3231; Stem Cell Technologies) and cultured for 2 days in the presence of 1 unit/ml erythropoietin (generous gifts from Chugai Pharmaceutical Co.). For BFU-E assays, 1 × 10^5 cells were plated in 1 ml of methylcellulose medium and cultured for 7 days in the presence of 2 units/ml erythropoietin and 100 ng/ml stem cell factor (generous gifts from Kirin Brewery). Colonies were stained with benzidine, and benzidine-positive colonies were counted.

Reverse Transcription-PCR—Total RNA was extracted from the peripheral blood cells of E12.5 embryos using ISOGEN (Nippon-gene) and then treated with RNase-free DNase (Promega). The cDNA was synthesized using SuperScriptTMII reverse transcriptase (Invitrogen) and random primer. To detect the expression of Bcl-XL, PCR amplification was performed as previously described (18). The expression of EpoR (erythropoietin receptor) was detected using the sense primer 5’-AGGCCCTCTCCTTTAACTCTGGGGA-3’ and the antisense primer 5’-AACTCGATGGTCCTGCCCTTACA-3’; Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control.

RESULTS

Gata1 Knockdown Mutants Harboring the G1HRD-GATA-4 Transgene Die in Utero—To examine whether GATA-4 can substitute for GATA-1 when expressed under the GATA-1 gene regulatory cassette, we generated transgenic mice expressing GATA-4 under the control of GIHRD (Fig. 1A). We successfully established four transgenic lines, and two of these lines (Lines 2 and 4) were used in this study. To identify the production of transgene-derived GATA-4 protein, immunoblot analysis was performed using fetal livers at E12.5 (Fig. 1B). The GATA-4 protein level in fetal liver was comparable between Lines 2 and 4. The endogenous GATA-4 protein observed in the heart was similar in wild type and these two transgenic lines (Fig. 1B). It is of note that the expression level of GATA-4 transgene in fetal liver was comparable with that of endogenous GATA-4 in the heart. These transgenic mice were born at the expected frequency and were fertile. Peripheral blood counts of these transgenic mice were within the normal range in the adult stage (data not shown).

We next examined whether the expression of GATA-4 under the control of GIHRD could rescue Gata1.05/Y embryos from lethal anemia. To this end, Gata1.05/X females were crossed with GIHRD-GATA-4 transgenic males, and the genotypes of the progeny were determined by PCR (Table 1). No male mice bearing the Gata1.05 allele were obtained from several litters in both transgenic lines, indicating that Gata1.05/Y embryos die in utero even in the presence of the GATA-4 transgene.

The genotypes of Line 2 and Line 4 embryos from E11.5 to E16.5 were examined (Table 2). Although in the absence of transgene all Gata1.05/Y mutants were found dead by E12.5, the majority of the Gata1.05/Y::GATA-4-TG compound mutants were alive at this stage. Furthermore, we observed nine Gata1.05/Y::GATA-4-TG embryos that survived until E13.5 and E15.5. These results thus demonstrate that, although the expression of GATA-4 is not sufficient for rescuing Gata1.05/Y mice from embryonic lethality, this substitution recapitulates the function of GATA-1 to some extent in the Gata1.05/Y embryos. Therefore, we referred to the surviving Gata1.05/Y embryos bearing GATA-4-TG (Gata1.05/Y::GATA-4-TG) as GATA-4-rescued embryos (G4R).

Primitive Erythroid Cell Maturation Is Impaired in G4R—We observed primitive erythropoiesis in G4R embryos. At E8.5, primitive erythroid cells were observed in blood islands of the yolk sac. The gross appearance of the yolk sac (Fig. 2, A, E, and I) and hematocrit and eosin staining of the sectioned blood islands (Fig. 2, B, F, and J) were similar in wild type, Gata1.05/Y, and G4R embryos. However, immunohistochemical analysis revealed that a large number of cells were positive for embryonic globin in wild type and G4R embryos (Fig. 2, C and K),
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The expression of the transgene (Fig. 2L). These results indicate that transgenic GATA-4 substituted for GATA-1 and induced embryonic globin gene expression at the early embryonic stage.

At E12.5, G4R embryos (Fig. 3B) were easily distinguishable from wild type embryos (Fig. 3A) by their anemic appearance. The majority of the peripheral blood cells harbored basophilic or polychromatic cytoplasm and large nuclei in the G4R embryos (Fig. 3D), whereas those of wild type embryos were mature primitive erythroid cells showing orthochromatic cytoplasm and small nuclei (Fig. 3C). Taken together, these results indicate that, whereas the expression of embryonic ε-globin is rescued, both cytoplasmic and nuclear maturation is impaired in the G4R primitive erythroid cells.

Maturation Arrest of Definitive Erythroblasts in G4R Fetal Liver—
At E12.5, the number of fetal liver cells in G4R embryos was less than one-third of wild type embryos (Fig. 4A), and, accordingly, G4R livers were much smaller than those of wild type (data not shown). A colony assay demonstrated a significant reduction in CFU-E formation in G4R embryos compared with wild type, whereas in contrast, the number of BFU-E colonies was comparable between G4R and wild type embryos (Fig. 4B). These results indicate that the progression of BFU-E to CFU-E is significantly impaired in G4R embryos.

We further assessed the maturation stage of fetal liver erythroid cells by flow cytometry (FACS). The maturation status of erythroid cells has been characterized by the expression profiles of c-Kit, CD71, and Ter119 (19, 20). Proerythroblasts are characterized as the c-Kit−Ter119− cell fraction. These cells differentiate into c-Kit−Ter119+ erythroblasts during maturation. The FACS profile of fetal liver cells demonstrated that this process was severely impaired in G4R embryos (Fig. 4C).

Moreover, the frequency of the Ter119−CD71− fraction that corresponds to proerythroblasts was increased in G4R embryos compared with wild type embryos (Fig. 4D, R2 fraction). Instead, the Ter119+CD71+ fraction corresponding to basophilic erythroblasts was reduced in G4R embryos compared with wild type (Fig. 4D, R3 fraction). Collectively, these FACS profiles demonstrate that erythropoiesis differentiation is impaired at the proerythroblast stage in G4R embryos. Consistent with these results, Wright-Giemsa staining of fetal liver cells revealed that G4R erythroid cells are larger in size and with larger nuclei than wild type erythroid cells (Fig. 4E). At E14.5, the majority of circulating erythrocytes were enucleated in wild type embryos, whereas almost all circulating erythrocytes were nucleated in G4R embryos (Fig. 4F).

whereas very few cells were positive in the yolk sac of the Gata1.05/Y embryo (Fig. 2G). GATA-4 immunostaining was positive in the blood island cells of the G4R embryo, confirming

FIGURE 3. Terminal maturation of primitive erythroid cells is impaired in G4R embryos. A and B, E12.5 wild type (A) and G4R (B) embryos. Note that G4R embryo is anemic in appearance compared with wild type embryo. C and D, Wright-Giemsa staining of peripheral blood cytospin cells prepared from E12.5 embryos. The primitive erythrocytes of wild type (C) are the mature type. In contrast, primitive polychromatic erythroblasts are observed in G4R embryos (D). Scale bars, 10 μm.

FIGURE 2. Embryonic globin is expressed in the G4R primitive erythroid cells. Shown is the gross appearance of E8.5 embryos (A, E, and I) and hematoxylin and eosin staining of E8.5 yolk sacs (B, F, and J) from wild type (A and B), Gata1.05/Y (E and F), and G4R (I and J) embryos. Shown is immunohistochemical analysis of E8.5 yolk sacs using anti-ε-globin antibody (C, G, and K) and anti-GATA-4 antibody (D, H, and L). The embryos are wild type (C and D), Gata1.05/Y (G and H), and G4R (K and L). Scale bars, 100 μm (B and C) and 10 μm (D).
These data demonstrate that in G4R embryos erythroid differentiation is affected at multiple stages of definitive erythropoiesis, suggesting that the expression of multiple genes may be attenuated in the G4R embryos. Therefore, it seems likely that maturation arrest of definitive erythroid cells in G4R embryos consequently results in defective expansion of fetal liver hematopoiesis and lethal anemia in the following gestation period.

**Transgenic Mouse Expressing GATA-4-GATA-1 Chimeric Protein**—The data obtained so far show that, whereas the G1HRD-GATA-2 or -GATA-3 transgene could rescue Gata1.05/Y embryos from lethal anemia (8), the G1HRD-GATA-4 transgene could not, suggesting the presence of unique functional properties of hematopoietic GATA factors that cannot be replaced by endodermal GATA factors. To delineate the molecular basis of the differences between hematopoietic and endodermal GATA factors, we focused on the zinc finger region of the GATA factors, because there is divergence outside this region even among the hematopoietic GATA factors. Alignment of the zinc finger region sequences of six murine GATA factors revealed that 12 amino acid residues are conserved in hematopoietic but not in endodermal GATA factors (Fig. 5, yellow). These residues are frequently located in the adjacent region of the C-terminal zinc finger (CF).

Therefore, in order to examine the contribution of these hematopoietic factor-conserved residues to the erythropoiesis in vivo, we generated transgenic mouse lines bearing GATA-4-GATA-1 (G4G1) chimeric protein and carried out complement rescue analysis. In the chimeric G4G1 protein, the N-terminal region of GATA-4 (amino acid residues 1–251), including the N-terminal zinc finger (NF), is linked to the CF and adjacent C-terminal region of GATA-1 (amino acid residues 240–412; Fig. 6A). It turns out that 10 of the 12 residues described above are derived from the GATA-1 part or “hematopoietic” residues in this chimeric protein. To verify the protein expression, an HA tag was added to the C-terminal region of the G4G1 protein. This chimeric protein activated transcription of a GATA box-containing reporter when expressed in nonhematopoietic cultured cells (Fig. 6B) and bound to a consensus GATA sequence in gel mobility shift assays (data not shown). Two lines of G4G1 Chimeric Protein Promotes Maturation of Primitive Erythroid Cells—To examine the activity of the G4G1 chimeric protein in hematopoiesis, we crossed G1HRD-G4G1-HA transgenic mice with different expression levels (Lines 1 and 2; Fig. 6C) were established. Therefore, in order to examine the contribution of these hematopoietic factor-conserved residues to the erythropoiesis in vivo, we generated transgenic mouse lines bearing GATA-4-GATA-1 (G4G1) chimeric protein and carried out complement rescue analysis. In the chimeric G4G1 protein, the N-terminal region of GATA-4 (amino acid residues 1–251), including the N-terminal zinc finger (NF), is linked to the CF and adjacent C-terminal region of GATA-1 (amino acid residues 240–412; Fig. 6A). It turns out that 10 of the 12 residues described above are derived from the GATA-1 part or “hematopoietic” residues in this chimeric protein. To verify the protein expression, an HA tag was added to the C-terminal region of the G4G1 protein. This chimeric protein activated transcription of a GATA box-containing reporter when expressed in nonhematopoietic cultured cells (Fig. 6B) and bound to a consensus GATA sequence in gel mobility shift assays (data not shown). Two lines of G4G1 transgenic mice with different expression levels (Lines 1 and 2; Fig. 6C) were established. We also generated transgenic lines expressing wild type GATA-1 protein with an HA tag at the C-terminal end as controls.

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One of the most significant differences between G4R and G4G1R mice was the macroscopic appearance of E12.5 embryos. The gross appearance of G4G1R embryos at E12.5 was indistinguishable from wild type embryos (Fig. 7, A and B, respectively), which is in clear contrast with the anemic appearance of E12.5 G4R embryos (Fig. 3B). It has been shown that the majority of circulating erythrocytes at E12.5 are derived from primitive erythropoiesis, whereas definitive erythroid cells proliferate...
rapidly in fetal liver at this stage (21). Since G4R primitive erythroid cells showed impaired maturation at E12.5 (Fig. 3D), we examined whether the G4G1 protein promoted maturation of primitive erythroid cells using the peripheral blood of E12.5 embryos. Indeed, the morphology of peripheral blood from E12.5 embryos clearly differed among G4R, G4G1R, and wild type embryos. In wild type and G1R embryos, nearly 80% of cells showed typical features of matured primitive erythrocytes with orthochromatic cytoplasm and small nuclei (Fig. 7, C and F, respectively). In contrast, almost all G4R erythroid cells had a basophilic cytoplasm and large nuclei (Fig. 7E).

In G4G1R embryos, the majority of the cells showed orthochromatic cytoplasm, but the frequency of polychromatic erythroblasts was slightly higher than in wild type embryos (Fig. 7D). Notably, the orthochromatic cells in G4G1R blood (Fig. 7D) were distinguishable from wild type (Fig. 7C) by the appearance of nuclei. In G4G1R cells, chromatin condensation was insufficient, and the size of nuclei (relative to cytoplasm) was larger than that of wild type cell nuclei.

We also categorized the peripheral blood cells by counting each class of erythroblasts. Consistent with the morphological examination, G4R embryos contained many polychromatic erythroblasts, whereas wild type and G1R embryos contained predominantly orthochromatic erythroblasts (Fig. 7G). G4G1R embryos harbored many orthochromatic erythroblasts with larger nuclei (Fig. 7G, hatched columns). These results thus indicate that the G4G1 chimeric protein supports the differentiation of primitive erythroid cells much more dramatically than GATA-4 does.

G4R Primitive Erythroid Cells, but Not G4G1R Cells, Undergo Apoptosis—In addition to the delay in cell maturation, we frequently found nuclear fragmented cells in the G4R peripheral blood cells at E12.5 (Fig. 7, E and H, arrows). These cells were also observed in G4G1R, albeit at a much lower frequency (Fig. 7H). To examine whether these cells are apoptotic cells, the expression of Annexin V, a marker of apoptosis, was examined by FACS (Fig. 8A). Indeed, the frequency of Annexin V-positive cells was significantly greater in G4R cells than in wild type or G4G1R cells. The expression of Ter119 in the Annexin V-positive cells was negative or at a low level, indicating that these apoptotic cells were immature erythroblasts.

To examine how apoptosis increased in the G4R primitive erythroid cells, we examined the expression of Bcl-X and EpoR, the two antiapoptotic genes expressed in erythroid cells. These two factors have been shown to control the survival of erythroid cells in distinct stages of differentiation. In the early stage of differentiation, Epo-EpoR signaling predominantly acts to prevent apoptotic cell death (22), whereas Bcl-XL acts at the later stage of erythroid cell maturation (23–25). The reverse transcription-PCR analysis revealed that the expression of Bcl-XL was significantly reduced in the G4R cells, whereas that of EpoR was unaffected (Fig. 8B). Moreover, the expression of Bcl-XL was slightly reduced in the G4G1R cells compared with wild type cells, consistent with the mild increase of the Annexin V-positive cells (Fig. 8A). These results suggest that both GATA-4 and the G4G1 chimeric proteins fail to sustain the expression of Bcl-XL in primitive erythroid cells. The lower frequency of apoptosis in the G4G1R cells compared with the
G4R cells suggests that there may be a threshold level of Bcl-XL needed for the erythroid cell survival.

The G4G1 Chimeric Protein Fails to Fully Support Definitive Erythropoiesis in Fetal Liver—In contrast to the primitive erythropoiesis, the G4G1 chimeric protein failed to sustain definitive erythropoiesis in fetal liver, although G4G1R embryos survived longer than G4R. A colony assay revealed that the number of CFU-E, but not BFU-E, was reduced in the G4G1R fetal liver cells at E13.5 compared with wild type and G4G1Tg embryos (Fig. 9A). FACS analysis demonstrated that the expression level of Ter119 is clearly decreased in the CD71/H11001 fraction of the G4G1R liver compared with that of wild type embryos, indicating that maturation of definitive erythroid cells is impaired in the G4G1R fetal liver.

DisCUSSION

In this study, we have attested the functional incompatibility between hematopoietic and endodermal GATA factors in vivo. Using the transgenic complementation rescue approach, we have demonstrated that GATA-4 is unable to fully replace the functions of GATA-1 in both primitive and definitive erythropoiesis in mouse. Although the phenotype of G4R is less severe than that of Gata1.05/Y mice, the maturation of definitive erythroid cells is arrested in G4R fetal liver, leading to embryonic death by E15.5. In stark contrast, we previously demonstrated that GATA-2 and GATA-3 expressed from the same gene-regulatory cassette could compensate for the absence of GATA-1 and eliminate lethal anemia in Gata1.05/Y embryos (8). These data suggest that the hematopoietic GATA factors can promote embryonic erythropoiesis through conserved biological properties that are absent in the endodermal GATA factor GATA-4.

It is interesting to note that GATA-4 rescued erythroid cell differentiation when introduced into embryoid bodies lacking GATA-1 (26). GATA-4 induced the expression of ζ-, β-, and α-globin genes in GATA-1-null embryonic bodies to levels similar to those induced by GATA-1. In this study, we found that ε-globin expression was rescued in the G4R yolk sac. These results suggest that GATA-4 can replace GATA-1 function to induce globin gene expression. Importantly, however, our study also revealed that the expression of a set of erythroid genes requires the specific contribution of hematopoietic GATA factors, which cannot be replaced by GATA-4 in vivo.

We further exploited the transgenic complementation rescue approach to pinpoint the GATA-1 regions responsible for supporting embryonic erythropoiesis. When analyzed in this
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FIGURE 8. G4R primitive erythroid cells undergo apoptosis. A, FACS analysis of E12.5 peripheral blood using anti-Ter119 and Annexin V. Annexin V-positive cells were observed in G4R but much less in G4G1R and wild type embryos. B, semiquantitative reverse transcription-PCR analysis of E12.5 primitive erythroid cells. Note that the expression level of Bcl-X_L is decreased in G4R embryos, whereas the expression of EpoR is unaffected.

system, the G4G1 chimeric protein can promote the differentiation and survival of primitive erythroid cells, albeit with incomplete nuclear maturation during the orthochromatic erythroblast stage. These results indicate that the CF and adjacent region of GATA-1 play important roles in primitive erythropoiesis that cannot be carried out by the corresponding region of GATA-4. The results are quite intriguing, because the function of the C-terminal region of GATA-1 in vivo has been largely unknown.

The CF domain of GATA factors is essential for DNA binding. The CF domain also provides an interface for protein-protein interactions with a variety of transcription factors and cofactors, such as EKLF, Lmo2, PU.1, and CREB-binding protein/p300 (27–30). Since erythropoiesis-promoting activities are clearly different between hematopoietic GATA factors and GATA-4, we focused on 12 amino acid residues within the zinc finger domains that are conserved among the hematopoietic GATA factors but not in GATA-4. In the G4G1 chimeric protein, 10 of the 12 residues originated from GATA-1 (referred to as hematopoietic residues). Some of these residues are reported to possess specific biochemical properties. Although we still cannot pinpoint the critical difference between hematopoietic and endodermal GATA factors, we feel that these properties are critical for the specific function of GATA-1 in vivo. The following two observations may be pertinent for understanding the difference between hematopoietic and endodermal GATA factors in relation to the 12 divergent amino acid residues.

First, we noticed that lysine residues 245 and 315, which have been shown to be involved in the self-association of GATA-1 (31), are among the 10 hematopoietic residues in the G4G1 chimeric protein. We previously demonstrated that replacement of six lysine residues with alanine in zebrafish GATA-1 has been shown to exhibit higher DNA binding activity than the nonacetylated form (34). These two motifs are highly conserved in hematopoietic GATA factors but not in endodermal GATA factors. Especially, the C-terminal KGKK motif, mapped as the major site of acetylation by CREB-binding protein (33), corresponds to the quite distinct sequence LNKS in GATA-4. However, a recent study showed that GATA-4 is acetylated during the differentiation of embryonic stem cells into cardiac myocytes (35). Taken together, these results suggest that protein-protein interactions and post-translational modifications of the GATA proteins in vivo require a specific cellular context.

We observed impaired maturation of primitive erythroid cells at distinct stages of differentiation in Gata1.05/Y, G4R, and G4G1R embryos, indicating that GATA-1 plays prominent roles in multiple stages of cell maturation during primitive erythropoiesis. Several transcription factors besides GATA-1 have also been reported to promote primitive erythropoiesis. In particular, EKLF and Gfi-1b play important roles in the maturation of primitive erythroid cells (36–38). Importantly, GATA-1 physically interacts with these factors through zinc finger regions (27, 39). Mice lacking EKLF die around E14 due to defective definitive erythropoiesis (40, 41). EKLF-null primitive erythroid cells are irregularly shaped and contain inclusion bodies resulting from the precipitation of free globins. These abnormalities are partly caused by a reduction in erythroid membrane protein band 4.9 and α-hemoglobin-stabilizing protein (36, 37). Gfi-1b is a zinc finger proto-oncogene product essential for the development of erythroid and megakaryocytic lineages (38, 42). Gfi-1b-null primitive erythroid cells showed a maturation defect in primitive erythroid cells, characterized by large nuclei and basophilic cytoplasm (38). It is interesting to note that the defective maturation seen in G4R primitive eryth-
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One of the significant differences between G4R and G4G1R primitive erythroid cells is the incidence of apoptosis. Apoptotic primitive erythroid cells in the immature stage were increased in G4R embryos, whereas the frequency of apoptosis was much lower in G4G1R embryos. We found that expression of the Bcl-X gene was reduced significantly in G4R cells and mildly in G4G1R cells compared with wild type cells, whereas that of EpoR was unaffected in these mutants. Curiously, in cardiomyocytes, GATA-4 induces the Bcl-X gene and promotes cell survival (43). Therefore, a specific cellular context is presumably important for GATA-4 in promoting cell survival and activation of the Bcl-X gene.

Although G4G1R embryos survive longer than G4R, maturation arrest of definitive erythroid cells was observed in the G4G1R fetal liver at E13.5. The extended life span of the G4G1R embryos could be explained, at least in part, by the improved primitive erythropoiesis of the G4G1R yolk sac compared with that of G4R. In addition, it is plausible that the G4G1 chimeric protein may work better than GATA-4 to support proliferation and survival of definitive erythroid cells, although the protein seems not to bear a significant advantage in supporting the erythroid maturation. Therefore, the erythropoiesis in the G4G1R embryos appears not so effective as to fully support the postnatal erythropoiesis. These results thus indicate that the N-terminal region of GATA-1 is necessary to support the definitive erythropoiesis in addition to the C-terminal half.

In summary, this study demonstrates the incomplete substitution by GATA-4 of GATA-1 function during embryonic erythropoiesis. Specific biological properties reside in hematopoietic GATA factors that cannot be replaced by endodermal GATA factors. Phenotypic differences among G4R, G4G1R, and G1R illustrate the function of GATA-1 at multiple stages during primitive erythroid cells resembles that in Gfi-1b-null embryos rather than that in EKLF-null embryos. Thus, GATA-4 may be incapable of interacting with some GATA-1 partners, leading to an attenuation of gene expression that requires a specific interaction of GATA-1 with a partner protein.
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In addition, the results also suggest specific roles of the C-terminal region of GATA-1. We conclude that this approach is a promising one for dissecting the functional subdomain of GATA-1 for its specific biological function in vivo.

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