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Arrested development of embryonic red cell precursors in mouse embryos lacking transcription factor GATA-1

(embryonic stem cells/gene targeting/hematopoiesis)

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ABSTRACT **The X chromosome-linked transcription factor GATA-1 is expressed specifically in erythroid, mast, megakaryocyte, and eosinophil lineages, as well as in hematopoietic progenitors. Prior studies revealed that gene-disrupted GATA-1$^-$ embryonic stem cells give rise to adult (or definitive) erythroid precursors arrested at the proerythroblast stage *in vitro* and fail to contribute to adult red blood cells in chimeric mice but did not clarify a role in embryonic (or yolk sac derived) erythroid cells. To examine the consequences of GATA-1 loss on embryonic erythropoiesis *in vivo*, we inactivated the GATA-1 locus in embryonic stem cells by gene targeting and transmitted the mutated allele through the mouse germ line. Male GATA-1$^-$ embryos die between embryonic day 10.5 and 11.5 (E10.5–E11.5) of gestation. At E9.5, GATA-1$^-$ embryos exhibit extreme pallor yet contain embryonic erythroid cells arrested at an early proerythroblast-like stage of their development. Embryos stain weakly with benzidine reagent, and yolk sac cells express globin RNAs, indicating globin gene activation in the absence of GATA-1. Female heterozygotes (GATA-1$^{+/-}$) are born pale due to random inactivation of the X chromosome bearing the normal allele. However, these mice recover during the neonatal period, presumably as a result of *in vivo* selection for progenitors able to express GATA-1. Our findings conclusively establish the essential role for GATA-1 in erythropoiesis within the context of the intact developing mouse and further demonstrate that the block to cellular maturation is similar in GATA-1$^-$ embryonic and definitive erythroid precursors. Moreover, the recovery of GATA-1$^{+/-}$ mice from anemia seen at birth provides evidence indicating a role for GATA-1 at the hematopoietic progenitor cell level.**

Differentiation of hematopoietic cells is controlled in large part by lineage-specific transcription factors (1). Of these, GATA-1, a zinc-finger protein, serves critical functions in erythroid cell development (2, 3). GATA-1, the founding member of a small family of related transcription factors, recognizes a consensus motif [(T/A)GATA(A/G)] present in the regulatory elements of virtually all erythroid-expressed genes. Within the hematopoietic system, GATA-1 is expressed in multipotential progenitors as well as committed erythroid, mast, megakaryocyte, and eosinophil precursors. GATA-1 is expressed at high level in both yolk sac-derived (embryonic or primitive) and adult (definitive) erythroid precursors.

A requirement for GATA-1 in normal erythroid development has been established by gene targeting experiments in mouse embryonic stem (ES) cells (4). Since the GATA-1 locus is situated on the X chromosome (5), effects of disruption of the GATA-1 gene in male ES cells can be assessed directly in chimeric mice (4) or upon *in vitro* differentiation of ES cells into hematopoietic cells (6). The first evidence that GATA-1 is essential for normal erythropoiesis was derived from chimera experiments in which it was shown that GATA-1 null (GATA-1$^-$) ES cells fail to contribute to mature adult red cells (4). Study of *in vitro* differentiated ES cells, as well as hematopoietic progenitors from chimeric mice, demonstrated that definitive erythroid precursors, indeed, form in the absence of GATA-1 but are arrested at the proerythroblast stage (6, 7) and die thereafter by apoptosis (8). Within arrested GATA-1$^-$ proerythroblasts, numerous putative GATA-1 target genes, including globins, are expressed, presumably under the aegis of the related protein GATA-2, which is ≈50-fold more abundant in GATA-1$^-$ versus normal proerythroblasts (6). Prior studies failed to detect GATA-1$^-$ embryonic erythroid precursors *in vitro* either due to a block in commitment of progenitors to an erythroid pathway or an inability to culture mutant embryonic precursor cells. Therefore, whether the requirement for GATA-1 in embryonic and definitive cells differs has remained uncertain.

To address the role of GATA-1 in embryonic erythropoiesis and also examine the phenotype of GATA-1 deficiency *in vivo*, we have transmitted a mutated GATA-1 allele through the germ line. Examination of GATA-1$^-$ embryos reveals the presence of developmentally arrested embryonic proerythroblast-like cells, thereby establishing a similar defect in both GATA-1$^-$ embryonic and definitive erythroid precursors. The generation of mice containing a germ-line mutation at the GATA-1 locus should permit production of compound mutant embryos to test the potential overlapping and compensatory roles of GATA-1 and GATA-2 in early hematopoiesis.

MATERIALS AND METHODS

Construction of Targeting Vector. GATA-1 genomic fragments isolated from a mouse strain 129Sv library (Stratagene) (4) were used to construct the targeting vector. The 5′ homology region was provided by a 2.2-kb *Nco*I fragment containing intron sequences and terminating at the initiator codon. The 3′ homology region was contained within a 3-kb *Eco*RI fragment including exons 3–6. Sequences between the initiator codon (exon 2) and the *Eco*RI site in exon 3 were replaced by a phosphoglycerate kinase-promoter-driven neomycin-resistance gene cassette. A herpes simplex virus thymidine kinase (HSV TK) cassette was added at the boundary of the construct for negative selection (9). The fragments were cloned into a synthetic polylinker to facilitate assembly of the targeting vector.

Gene Targeting and Generation of GATA-1$^-$ Embryos. The targeting construct was linearized by digestion with *Cla*I and electroporated into J1 ES cells (10). DNAs of colonies resistant to G418 and ganciclovir were subjected to Southern blot

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Abbreviations: ES, embryonic stem; E, embryonic day(s); CFU-e, erythroid colony-forming unit(s); BFU-e, erythroid blast-forming unit(s); Epo, erythropoietin.

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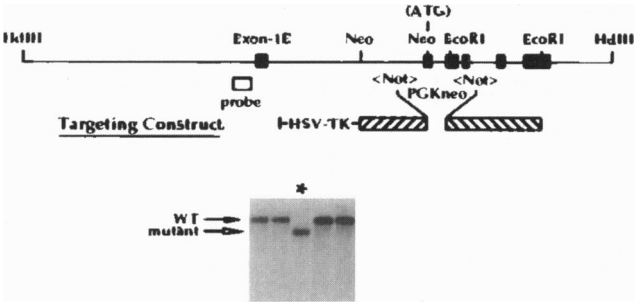

FIG. 1. Targeted disruption of the GATA-1 locus in ES cells. A schematic map of the GATA-1 locus is shown at the top with the targeting construct below. In the targeting plasmid a PGK-neo cassette was introduced at artificial *Not*I restriction sites (<Not>) to replace coding sequences including and downstream of the initiator codon. Below is shown a Southern blot of *Hin*dIII-digested DNA from G418/ganciclovir-resistant ES cell clones. The wild-type GATA-1 fragment is approximately 18 kb in size. The targeted clone is indicated by the asterisk.

analysis (11) using *Hin*dIII digestion and a 500-bp PCR-generated fragment from the GATA-1 promoter. Targeted clones were microinjected into host blastocysts derived from C57BL/6 mice. Chimeric animals were bred to C57BL/6 mice to obtain GATA-1 heterozygous F_1 mice. F_1 heterozygous females were bred to C57BL/6 males to obtain GATA-1$^-$ embryos. Genotyping of embryos was performed by PCR with the following neo primers: GCCCGGTTCTTTTTGTCAAGACC and CAGAAGAACTCGTCAAGAAGGCGA.

Histological Analysis. Embryonic day (E) 9.5 yolk sac and E10.5 embryos were fixed in Bouin's solution (Sigma) and embedded in paraffin. Sections were cut at 5 μm thickness, mounted, and stained with hematoxylin/eosin. Cytocentrifuged preparations of blood cells from E10.5 embryos were stained with May–Grunwald/Giemsa stain.

Reverse Transcription-Coupled PCR Analysis. Total RNA was prepared from individual E9.5 yolk sacs and reverse-transcribed with oligo(dT) as primer. PCRs were performed using primers specific for hypoxanthine phosphoribrosyltransferase (HPRT), βH1-, α-, and ζ-globin, and GATA-1 transcripts in the presence of tracer [^{32}P]dCTP as described (6). GATA-1 primers were chosen from exon 2 (TCAGCACTGGCCTACTACAG) and exon 3 (TAAGCACTGCCGGTGACAGG). The former primer is included in the region deleted in the GATA-1$^-$ allele. All PCR products distinguished between genomic DNA and cDNA since primers were located in different exons.

***In Vitro* Progenitor Assay of Yolk Sac Cells.** Single cell suspensions of E9.5 yolk sac cells were prepared by digestion with 0.1% collagenase (Sigma)/20% fetal calf serum (FCS; HyClone) in minimal essential medium (GIBCO/BRL) at 37°C for 1 hr. Cells were plated in medium containing 0.8% methylcellulose, 30% FCS, 1% bovine serum albumin, 2 mM L-glutamine, and 0.1 mM 2-mercaptoethanol (Methocult M3230; Stemcell Technologies, Vancouver) supplemented with penicillin/streptomycin. For the erythroid colony-forming unit (CFU-e) assay, erythropoietin (Epo) alone was added to the medium. For the erythroid blast-forming unit (BFU-e) assay, Epo and kit ligand were added. CFU-e and BFU-e colonies were examined after 3–4 days and 7–10 days, respectively. Cytocentrifuged cell preparations were stained with May–Grunwald/Giemsa stain.

Benzidine Staining of Whole Mount Embryos. E9.5 embryos were dissected free of extraembryonic membranes and benzidine staining was performed as described (12). Photographs were taken using Kodak EPY64T film under 5.5× magnification.

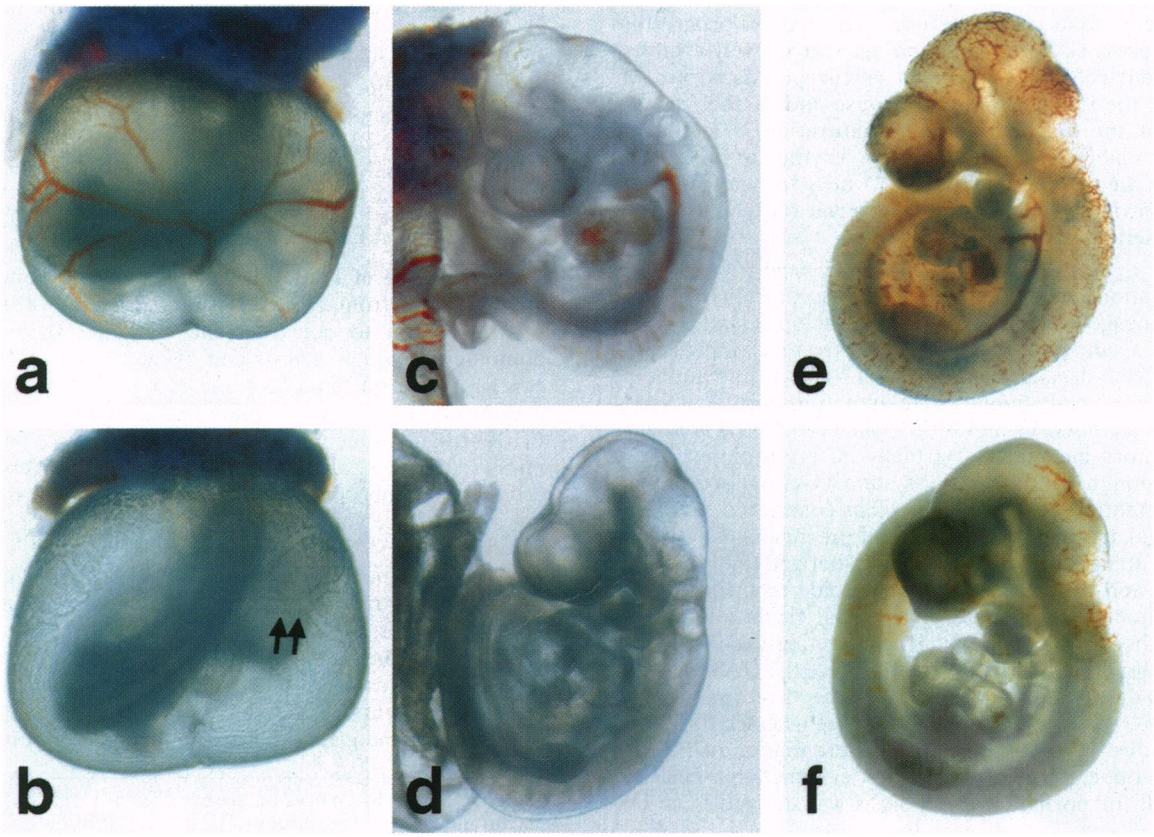

FIG. 2. Phenotype of wild-type and GATA-1$^-$ yolk embryos. Wild-type (*a*) and GATA-1$^-$ (*b*) E9.5 embryos with intact yolk sac membranes. The arrow indicates yolk sac vasculature. Wild-type (*c*) and GATA-1$^-$ (*d*) E9.5 embryos. Wild-type (*e*) and GATA-1$^-$ (*f*) E9.5 embryos stained with benzidine reagent.

RESULTS

Targeted Disruption of the GATA-1 Gene. A targeting vector designed to replace sequences extending from the initiator codon of the GATA-1 gene to an *Eco*RI site in exon 3 with a neomycin-resistance cassette was introduced into J1 ES cells (9) (Fig. 1). Approximately 1% G418/ganciclovir-resistant clones underwent an homologous recombination event, as shown by Southern blot analysis (Fig. 1). Among high-percentage coat color chimeras generated with GATA-1$^-$ ES cells, the majority transmitted the mutation to a small fraction of progeny upon mating with C57BL/6 mice (0–20% of offspring), except for one 80% chimera that transmitted to all progeny. The majority of F_1 heterozygous (GATA-1$^{+/-}$) female mice were noted to be pale at birth and during the neonatal period. In light of the failure of GATA-1$^-$ ES cells to contribute to adult red cells in chimeras (4), we interpret the anemia observed in heterozygotes to reflect random X chromosome inactivation of the normal allele in female cells. As heterozygous females age, anemia is no longer evident (data not shown), presumably due to *in vivo* selection of progenitor clones with an active normal allele.

Phenotype of GATA-1$^-$ Embryos. Male GATA-1$^-$ embryos were obtained by breeding GATA-1$^{+/-}$ F1 females with C57BL/6 males. At E9.5 and E10.5, mutant embryos were comparable in overall development and somite number to wild-type littermates but were notable for extraordinary pallor (Fig. 2 *a* and *c* versus *b* and *d*). Examination of embryos and yolk sacs revealed normal organization of the vasculature (Fig. 2 *a* and *b*). Although no red blood was evident in the vessels of GATA-1$^-$ embryos upon gross inspection (Fig. 2*b*), whole embryos stained weakly positive with benzidine, indicative of the presence of some heme (or hemoglobin)-containing blood cells (Fig. 2 *e* and *f*). Histological analysis of yolk sac sections (E9.5) revealed hematopoietic cells within the yolk sac blood islands (Fig. 3 *a* and *b*). Histological examination of E9.5 and E10.5 GATA-1$^-$ embryos was otherwise unremarkable (data not shown). GATA-1$^-$ embryos die between E10.5 and E11.5 due to severe anemia.

Arrested Embryonic Erythropoiesis in the Absence of GATA-1. Blood cells were recovered from E10.5 embryos for morphological examination. At this stage, a population of maturing embryonic erythroblasts is evident in wild-type embryos (Fig. 3*c*). In contrast, embryonic red cells of GATA-1$^-$ embryos are arrested at the proerythroblast stage and also display occasional nuclear fragmentation (Fig. 3*d*). Thus, GATA-1$^-$ embryonic red cell precursors form *in vivo* but are unable to complete their maturation. This phenotype closely resembles that observed in definitive erythropoiesis *in vitro*.

Hematopoietic Colony Formation from GATA-1$^-$ Yolk Sacs. Definitive hematopoietic colonies can be obtained upon *in vitro* culture of yolk sac cells. Single cell suspensions were prepared from yolk sacs and cultured in methylcellulose supplemented with Epo and kit ligand. CFU-e colonies, grown in Epo alone, were readily obtained from wild-type yolk sacs. In contrast, GATA-1$^-$ yolk sacs yielded only rudimentary colonies that were not red and contained dying cells (data not shown). Wild-type colonies grown in Epo plus kit ligand displayed BFU-e-like morphology and contained erythroid and other cell types (Fig. 3 *e* and *g*), whereas large pale colonies were obtained from GATA-1$^-$ yolk sacs (Fig. 3 *f* and *h*). The mutant colonies contained developmentally arrested dying proerythroblasts and abundant megakaryocyte-like cells that stained positively for acetylcholinesterase (data not shown). Yolk sacs from GATA$^{+/-}$ embryos yielded both types of colonies. Formation of myeloid colonies from GATA-1$^-$ embryos was not affected.

Globin Gene Expression in GATA-1$^-$ Embryos. Studies of GATA-1$^-$ ES cells differentiated *in vitro* into erythroid colonies have indicated that numerous presumptive GATA target genes are still expressed in the absence of GATA-1 (6). To determine whether globin genes are transcriptionally active in GATA-1$^-$ embryonic erythroblasts, total RNA was prepared

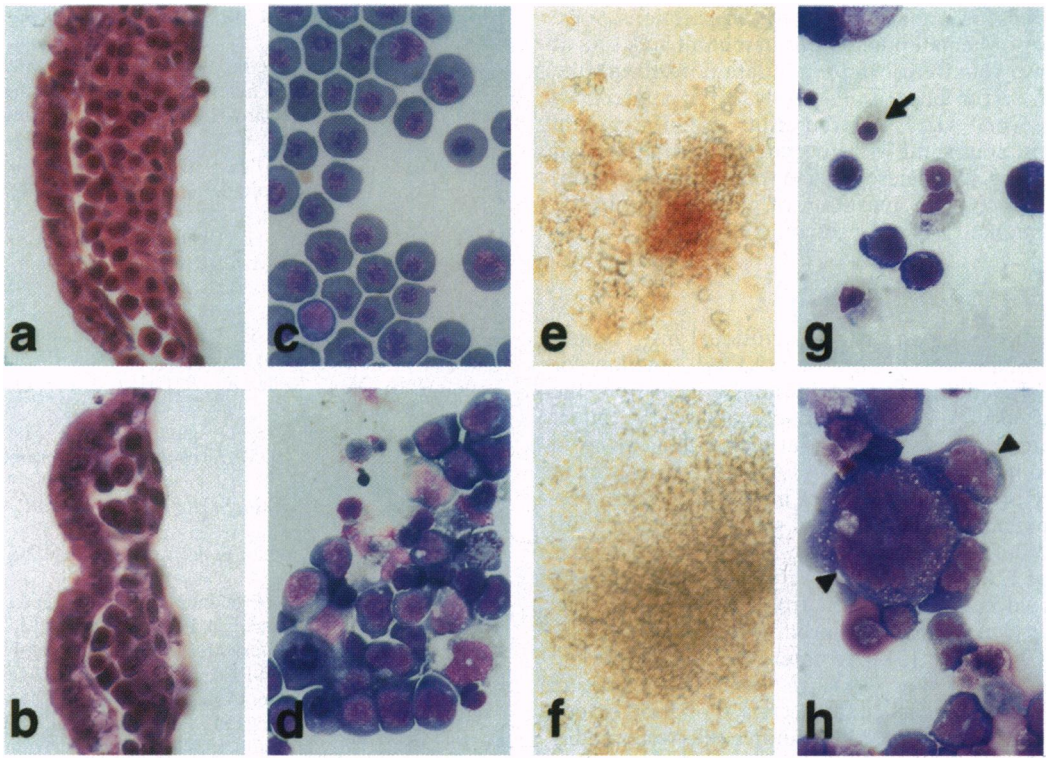

FIG. 3. Hematoxylin/eosin-stained sections of wild-type (*a*) and GATA-1$^-$ (*b*) E9.5 yolk sacs. Note the normal histology of blood islands and the presence of hematopoietic cells in the mutant. Wild-type (*c*) and GATA-1$^-$ (*d*) E10.5 blood stained with Wright–Giemsa. Morphology of colonies derived from wild-type (*e*) and GATA-1$^-$ (*f*) E10.5 yolk sac cells. Note the large appearance of the mutant colony. Cells of a wild-type (*g*) and GATA-1$^-$ (*h*) colony stained with May–Grunwald/Giemsa. Arrow, erythroid cells in *g*; arrowhead, megakaryocytic cells in *h*.

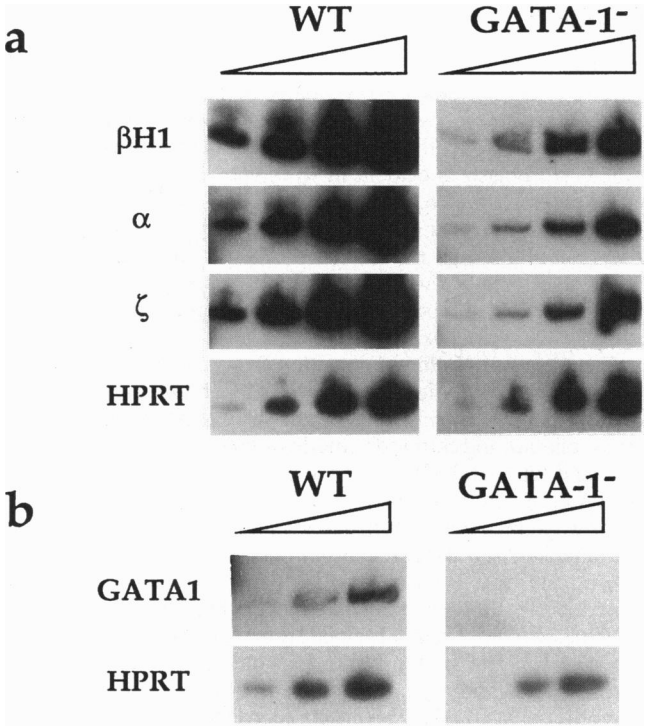

FIG. 4. Reverse transcription-coupled PCR analysis of yolk sac RNA. (*a*) Expression of globin gene (βH1, α-, and ζ-) transcripts in wild-type and GATA-1$^-$ E9.5 yolk sac cells. HPRT was used as a control. (*b*) Expression of GATA-1 transcripts in wild-type and GATA-1$^-$ E9.5 yolk sac cells. HPRT was used as control. The triangles depict increasing PCR cycles. *a*, 10–16 cycles; *b*, 18–24 cycles.

from E9.5 yolk sacs for semiquantitative reverse transcription-coupled PCR analysis. As shown in Fig. 4*a*, α-, ζ-, and βH1-globin transcripts were detected in GATA-1$^-$ samples, apparently at levels lower than in wild-type preparations. Since the total number of hematopoietic cells in mutant yolk sacs at this stage is lower relative to wild-type (data not shown), the abundance per erythroblast is not likely to be appreciably reduced. No normal GATA-1 transcripts are detected in mutant embryos, consistent with the targeted mutation that was introduced (Fig. 4*b*). These reverse transcription-coupled PCR experiments confirm activation of globin genes in the absence of functional GATA-1 protein.

DISCUSSION

The studies reported herein provide additional insights into the *in vivo* consequences of loss of GATA-1 function for erythropoiesis. In addition to establishing conclusively the critical role of GATA-1 within the context of the intact developing mouse, we have shown that embryonic erythroid precursors form in the absence of GATA-1 but are arrested at an early proerythroblast-like stage of maturation. Thus, erythroid development is blocked at a similar stage in both embryonic and definitive precursors, which implies a similar requirement for GATA-1 in the two erythroid lineages. Embryonic erythroid colonies derived from wild-type ES cells differentiated *in vitro* resemble the products of late precursors, analogous to CFU-e (6, 13). We infer, therefore, that the inability to detect embryonic GATA-1$^-$ erythroid precursors in prior *in vitro* experiments (6) reflects a limitation of cell culture assays. In contrast, arrested definitive cells were readily identified *in vitro* (6), because they arise from an earlier, BFU-e-like precursor.

Our analysis of RNA expression in yolk sacs provides strong confirmation that globin gene transcription is activated in GATA-1$^-$ erythroid cells, a finding consistent with prior studies of *in vitro* differentiated ES cells (6). This is a notable finding in that it demonstrates that GATA-1 *per se* is not uniquely required for activation of globin loci. Two possibilities may be raised. On the one hand, the transcription of globin genes might be activated independent of the action of GATA-1 or any other GATA factor. We consider this possibility remote in light of the large body of data implicating GATA motifs in the regulatory elements of virtually all erythroid-expressed genes and their presence in all core elements of globin locus control regions (14). More likely, the related GATA factor GATA-2, whose expression is not appropriately down-regulated in GATA-1$^-$ proerythroblasts (6), substitutes in part for GATA-1 and allows for the activation of globin and other erythroid-expressed genes. Accordingly, development of GATA-1$^-$ hematopoietic cells to the proerythroblast stage, in the embryonic or definitive lineage, may rely on GATA-2. The expression of GATA-2 at high level and its critical functions within immature hematopoietic cells are both compatible with this possibility (15).

The findings described herein and previous data (6, 7) establish an essential role for GATA-1 in terminal erythroid maturation. The behavior of GATA-1$^{+/-}$ mice provides evidence indicating an effect of GATA-1 deficiency at the progenitor level. The majority of GATA-1$^{+/-}$ mice are born anemic and then recover in the neonatal period. Such *in vivo* selection for erythroid cells able to express GATA-1 must take place at the progenitor level. This signifies a competitive advantage for those progenitors with an active GATA-1 allele.

The generation of mice with a germ-line mutation at the GATA-1 locus should permit formal testing of functional overlap between GATA-1 and GATA-2 during early hematopoietic development. Specifically, production of compound mutant embryos obtained by interbreeding of GATA-1 and GATA-2 (13) knockout heterozygotes should define the contribution of GATA-2 to erythroid commitment occurring in the absence of GATA-1 and may also provide insights into the potential role of GATA-1 in compensating for the absence of GATA-2 in immature progenitor cells.

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