To elucidate the in vivo function of GATA-1 during hematopoiesis, we specifically disrupted the erythroid promoter of the GATA-1 gene in embryonic stem cells and generated germ line chimeras. Male offspring of chimeras bearing the targeted mutation were found to die by 12.5 days post coitus due to severe anemia while heterozygous females displayed characteristics ranging from severe anemia to normal erythropoiesis. When female heterozygotes were crossed with transgenic males carrying a reporter gene, which specifically marks primitive erythroid progenitors, massive accumulation of undifferentiated erythroid cells were observed in the yolk sacs of the GATA-1-mutant embryos, demonstrating that GATA-1 is required for the terminal differentiation of primitive erythroid cells in vivo.

The transcription factor GATA-1 (1–3) is expressed in erythroid, megakaryocytic, and mast cells (4, 5) as well as in the Sertoli cells of the testis (6). GATA-1 is encoded by the X chromosome (7), and two alternative promoters, which direct GATA-1 expression in distinct cell lineages, have been identified. The 5′ promoter specifies expression in Sertoli cells while a downstream promoter located between the first testis-specific exon and the common coding exons of the gene directs the expression of GATA-1 in erythroid cells (8).

Analysis of GATA-1-null embryonic stem (ES) cells generated by homologous recombination has revealed that the mutant cells do not contribute to the mature erythroid population in chimeric animals (9). These ES cells also failed to differentiate into mature erythroid cells in vitro (10–12), suggesting that GATA-1 is required for the erythroid differentiation of hematopoietic progenitor cells. However, previous attempts failed to produce germ line transmission of the GATA-1-null allele. Thus, the in vivo role of GATA-1 in erythropoiesis remains to be resolved.

In this study, we enfeebled the GATA-1 gene through a disruption of its hematopoietic promoter in ES cells and generated germ line chimeras to directly address the in vivo effects of the targeted mutation. Our results demonstrate that GATA-1 is required for the terminal differentiation of primitive erythroid cells in vivo, and this work reveals for the first time the consequence of GATA-1 function in primitive erythroid progenitors during development.

EXPERIMENTAL PROCEDURES

GATA-1 Gene Targeting—Genomic clones of the GATA-1 locus were isolated from a mouse 129 SVJ A/2J strain (Stratagene). A 5.1-kilobase 5′-fragment and 2.7-kilobase 3′-fragment, which contains the erythroid-specific first exon (IE exon), were cloned into a vector containing a neomycin-resistance gene (neo) cassette to generate the targeting construct. The resultant construct was linearized with HindIII and electroporated into E14 cells as described (13). Clones were selected in G418 (300 μg/ml), expanded, and analyzed by PCR. Homologous recombination was observed at a frequency of ~1/10 G418-resistant clones. Chimeras were generated by injection of C57 BL/6 blastocysts as described previously. Pups and embryos were genotyped by PCR and/or Southern blotting analyses. We determined the sex of these embryos by using PCR amplification of Y chromosome-specific 252-bp-1 gene (14).

RT-PCR Analysis of Mouse Embryos—RNA from 9.5 days post coitus (dpc) embryos was isolated by single-step RNA extraction system (RNAzol, Tel-Test). First strand cDNA was synthesized using Superscript reverse transcriptase (Life Technologies, Inc.) at 37 °C for 1 h, and 1 μl of this 20-μl reaction mixture was used in PCR reactions. Reaction products were analyzed on 2% agarose gels. Sequences of the primers used and the expected sizes of PCR products are as follows: for erythroid-specific GATA-1, 5′-TAAAGGTGCTGATCTCTGATC and 3′-CCCTCTTCAAGTGTCCTCAAGAAGCT (expected product 483 bp); and for testis-specific GATA-1, 5′-CGTGAACGCGAGGATCGT and the 3′-primer used for the erythroid-specific primer set (expected product 529 bp). Primers for glucose-6-phosphate dehydrogenase were synthesized as described previously (expected product 162 bp; Ref. 15).

Histological Analysis of Newborn Mice and Embryos—Newborn mice were fixed in 10% buffered formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin for histological examination. Embryos were fixed after being dissected free of maternal tissues. β-Galactosidase (LacZ) activity was detected as described previously (16). After 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining, embryos were photographed before embedding in OCT compound (Sakura Finetechical) and freezing. Cryosections were processed for immunohistochemistry with anti-murine embryonic hemoglobin sera (17), which was kindly provided by Dr. Tadao Atsumi.

Fetal Liver Colony Assays—Fetal liver cells were isolated into a methyl cellulose culture in the presence of erythropoietin (2 units/ml) (18). After 2 days, the number of colonies formed were counted, colony forming unit-erythroid (CFU-E). Cells were also assayed for the ability to form granulocyte colonies, colony forming unit-granulocyte/macrophage (CFU-GM) in a semi-solid culture supplemented with granulocyte/macrophage colony stimulating factor, IL-3, and IL-6.

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RESULTS

Targeted Disruption of the Mouse GATA-1 Promoter—We prepared a positive/negative selection targeting vector to introduce a neo cassette into the intergenic region between an important regulatory region of the erythroid promoter and the mRNA cap site. GATA factor binding sites located approximately 620 bp 5' to the IE exon (Fig. 1A) have been shown to positively regulate transcription from the IE promoter (19). The insertion of neo was designed to separate the GATA motifs from the IE exon in the hope that the mutation would negatively affect the activity of the IE promoter. In addition, the positive/negative selection targeting vector was designed to transcribe neo in the same orientation as the GATA-1 gene so that the strong promoter directing neo expression might further weaken the activity of the IE promoter through competition (20, 21). Our expectation is that the activity of the IE promoter will be diminished by the combinatorial effects of these two mechanisms, resulting in a specific 'knock-down' (rather than a 'knock-out') of the erythroid-specific expression of the GATA-1 gene, and possibly lead to a germ line-transmissible targeted mutation.

This targeting construct was introduced by electroporation into ES cells, and 20 independent G418-resistant, PCR-positive clones were identified. Southern blot analysis using internal gene-specific and neo probes confirmed the homologous recombination events (Fig. 1B and C). Three mutant ES cell lines were used for the generation of chimeric offspring. All the ES cell lines gave rise to chimeras. We crossed these chimeras with normal Balb/c mice and one line (No. 78) gave stable germ line transmission. All F1 generation mice from the chimera mouse were found to be of ES cell origin, and the number of litters and sex ratio were in normal range.

Phenotype of Heterozygous Female Mice—F1 progeny were examined by Southern blot and PCR analyses to confirm the presence of the mutated GATA-1 allele. Since the GATA-1 gene is located on the X chromosome, all heterozygous F1 pups were female. These heterozygotes were found to display varying degrees of anemia. Fig. 2A shows GATA-1 heterozygous embryos; their appearance ranges from normal (left) to severely anemic (right). Concordantly, when we analyzed the livers of two heterozygous newborn littermates (panel B), we found that one liver contained a normal number of hematopoietic cells (left), whereas the other displayed a marked reduction in the number of hematopoietic cells (right). These observations indicated that disruption of the GATA-1 gene erythroid promoter seems to cause severe anemia in vivo and that the effect of the knock-down strategy was as anticipated. The heterogeneity of the phenotypes observed in the female heterozygotes may be the result of selective inactivation of the X chromosome (22).

Mutant Male Embryos Died In Utero Due to Impaired Primitive Erythropoiesis—The heterozygous females were mated with wild-type males to observe the phenotypic effects on mutant males. Surprisingly, no mutant males were observed among the live offspring, and heterozygous female offspring displayed the same varying degrees of anemia that we observed among the F1s. To determine the nature and timing of the presumptive embryonic lethality of the targeted males, we...
Promoter-specific Disruption of Mouse GATA-1 Gene

Decreased Number of Erythroid Cells and CFU-E in Mutant Fetal Livers—We examined the hematopoietic phenotype in the livers of mutant male embryos. Morphological examination revealed that size of the liver rudiments of mutant male embryos was markedly reduced and that the color was pale in comparison to the livers of wild-type embryos (data not shown). Microscopic analysis revealed that, whereas many erythroid cells should be present in the liver rudiment at 11.5 dpc, virtually none could be detected in the livers of mutant embryos (compare Fig. 5, panels A and B). This seems to reflect the absence of yolk sac hematopoiesis. GATA-1-positive cells were also absent in liver sections from 11.5-dpc GATA-1.05 mutant male (compare panels C and D).

We also performed in vitro colony assays with these same fetal liver cells. In comparison with wild-type littermates, the GATA-1.05 male mutant livers did not generate any CFU-E at 10.5 dpc (data not shown), and only a small number of CFU-E were recovered at 11.5 dpc (Table I). In contrast, fetal liver cells from either wild-type or mutant embryos produced similar numbers of CFU-GM colonies (Table II). These data indicate that only a small number of erythroid progenitors exist in the liver of the mutant embryos, while the non-erythroid hematopoietic lineages are unaffected by the mutation. The number of CFU-E formed from fetal liver cells of female heterozygous mutants (GATA-1(+/-1.05)) accordingly displayed wide variability, again consistent with the hypothesis that the X chromosome is selectively inactivated in hematopoietic progenitor cells.

GATA-1-deficiency Results in Accumulation of Primitive Erythroid Progenitors—To investigate how primitive hematopoiesis is affected by the lack of GATA-1, we took advantage of a GATA-1 promoter-LacZ transgene, which is transcriptionally active in primitive but not definitive erythroid cells, to specifically mark the primitive erythroid lineage (29). Female mice heterozygous for the GATA-1.05 mutation were crossed with transgenic mice homozygous for the LacZ reporter transgene to generate LacZ-positive embryos harboring the GATA-1.05 mutation (compound mutant designated GATA-1.05::LacZ).

In 8.0-dpc embryos examined by whole mount X-gal staining, no significant difference in either the abundance or distribution of stained erythroid cells was observed between GATA-1.05::LacZ mutant embryos and LacZ-only littermates (data not shown). By 9.5 dpc, however, the number of blue cells detected in the GATA-1.05::LacZ embryos was far greater than that seen in littermates carrying only the reporter transgene (compare panels A and B, Fig. 6). Detailed examination revealed that the absolute number of hematopoietic cells found in the yolk sac, aortic sac, and atrial chamber of the heart was...
similar in both wild-type and GATA-1.05 mutant embryos. The significant difference was that only a small percent of the hematopoietic cells in the atrial chamber were positive for staining in the LacZ control embryos (panel C), compared with the blue staining seen in virtually all of the hematopoietic cells in the GATA-1.05::LacZ male embryos (panel D). These blue GATA-1-positive cells express only a limited amount, if any, of embryonic hemoglobin as visualized by an embryonic hemoglobin anti-sera. It should be noted that, consistent with the whole mount analysis described above, virtually all hematopoietic cells in the yolk sac blood islands of 8.5-dpc LacZ transgenic mouse embryos were blue regardless of whether they carried the GATA-1.05 or wild-type allele (data not shown), confirming the specific expression of the reporter GATA-1/LacZ transgene in undifferentiated primitive hematopoietic progenitors. Therefore, in the GATA-1.05 mutant background, it seems that primitive hematopoietic progenitors develop to the GATA-1-positive stage but subsequently cannot differentiate into normal hemoglobinized cells.

**DISCUSSION**

We show here that a modified gene targeting strategy can be successfully employed to dissect the complex roles played by GATA-1 in vivo. This strategy consisted of two components. The first is promoter-specific impairment (knock-down) of transcription factor gene activity, and the second is the lineage- and stage-specific marking of the lineage of target cells that specifically require the function of the transcription factor. The combination of these two approaches enabled us to identify the precise stage of maturation arrest within the primitive erythroid lineage that resulted as a direct consequence of the lowered levels of GATA-1. The in vivo approach described here may be applied to the analysis of any gene of interest with well characterized regulatory regions.

We are able to detect the accumulation of primitive erythroid progenitors in the yolk sac of the GATA-1-mutant embryos, demonstrating that GATA-1 is necessary for the differentiation, but not the formation, of primitive erythroid progenitors. These results are especially intriguing and contrast with the conclusions of a previous report (24). Using in vitro differentiation of GATA-1-null ES cells, Weiss et al. (11) failed to detect primitive erythroid precursors, leading them to conclude that GATA-1 is essential at the earliest stage of primitive erythropoiesis definable by then current methods. In contrast, we observed numerous primitive erythroid progenitors in the yolk

**FIG. 5. Decreased number of erythroid cells in mutant fetal livers.** Hematoxylin and eosin staining of the liver sections of 11.5-dpc wild-type (A) and GATA-1.05 mutant male (B) embryos are shown. The arrowhead indicates a cluster of hemoglobinized cells. Anti-GATA-1 antibody staining of a 12.5-dpc wild-type embryonic liver section (C) and an 11.5-dpc GATA-1.05 male mutant (D) are also shown. The arrowhead indicates a GATA-1-positive cell.

**FIG. 6. GATA-1 deficiency causes accumulation of primitive erythroid progenitors.** X-gal staining of GATA-1 promoter-LacZ transgene in 9.5-dpc wild-type (A) and mutant male (B) embryos. The blue color produced by reaction with the β-galactosidase substrate can be observed in embryos of both genotypes, but the intensity detected in mutant males appears to be more prominent than that in wild-type embryos. The size of the GATA-1.05 mutant embryo (B) is much smaller than that of the wild-type embryo (A). X-gal staining was combined with anti-embryonic hemoglobin antibody staining on both types of embryos. While many embryonic hemoglobin antibody-positive cells are present in the atrial chambers of wild-type embryos (panel C, brown), few can be seen in the atrial chambers of mutant male embryos (D) even though numerous X-gal-positive (blue) cells are present. The arrowheads in panels A and C indicate hemoglobinized cells. Original magnifications are ×200 (panels C and D).

**TABLE I**

Results of the fetal liver CFU-E assays

| Genotype | Wild type | +/- female | -Y male |
|----------|-----------|------------|---------|
| Embryo number | 123456789  | 987654321  | 123456789 |

The numbers shown represent the colony number per liver ± S.E. from 11.5 dpc embryos. Assays were performed in triplicate. Colonies were counted 2 days after plating.

**TABLE II**

Results of the fetal liver CFU-GM assays

| Genotype | Wild type | +/- female | -Y male |
|----------|-----------|------------|---------|
| Embryo number | 123456789  | 987654321  | 123456789 |

The numbers shown represent the colony number per liver ± S.E. from 11.5 dpc embryos. Assays were performed in triplicate. Colonies were counted 6 days after plating.
sacs and atrial chambers of GATA-1.05 mutant embryos, and the erythroid enhancer of GATA-1 seems active in the progenitor cells. Two possibilities could account for this discrepancy. First, it is possible that a cell lacking GATA-1 may be able to survive in vivo but not under in vitro conditions (24). Alternatively, the residual 5% activity displayed by GATA-1 in the 1.05 mutant allele might be sufficient for cell survival but not for promoting terminal differentiation. We favor the former hypothesis since the knock-down effect on erythropoiesis seems to be clonal, and not uniform, in the erythroid cells (some weakly hemoglobinized cells in the yolk sac of the mutant embryos are routinely detected).

In this regard, it should be noted that Fujiwara et al. (25) recently reported the germ line transmission of a GATA-1-null allele. In that study, the second exon of the GATA-1 gene was disrupted in ES cells, as was the method employed in several previous trials (9–12), and thus the reasons for the failure to generate germ line chimeras using the GATA-1-disrupted ES cells prepared for previous studies remains unclear. In agreement with the present analysis, male mice bearing the GATA-1-null allele (i.e. /Y genotype) died by 11.5 dpc of embryogenesis. The coincidence of the null and knock-down mutant phenotypes suggests that the knock-down strategy resulted, as anticipated, in enfeebling the gene to such an extent that its final activity was very close to that of a null mutation, while analysis of the Sertoli GATA-1 transcript indicates that its synthesis, as also anticipated, is unaffected by the GATA-1.05 mutation. Fujiwara et al. (25) also found accumulation of embryonic erythroid cells in GATA-1-null embryos and suggested that the inability to detect embryonic erythroid precursors in prior in vitro experiments reflected a limitation of cell culture assays. Finally, they suggested that the accumulated cells in the yolk sac of the null mutant mouse were arrested at the proerythroblast stage based on morphological examination. In contrast, the present study provides direct evidence for the accumulation of primitive erythroid progenitors in the yolk sac blood islands through an intercross experiment performed by breeding the GATA-1.05 mutant allele to a separate line of mice bearing a transgene that specifically marked the primitive erythroid lineage. As additional evidence, we were able to unequivocally identify the arrested cells as primitive erythroid progenitors using both anti-GATA-1 and anti-embryonic hemoglobin antibodies.

In addition to the primitive erythroid lineage analysis, preliminary studies on the effect of GATA-1 mutation on fetal liver (definitive) erythropoiesis in vivo are presented here for the first time, and we also found this developmental process to be severely affected by the knock-down mutation. The mutant fetal livers were observed to contain only a small number of CFU-E. Since we detected comparable numbers of CFU-GM in either GATA-1(+/+) or GATA-1.05 mutant mice, the GATA-1 deficiency appears to specifically promote the growth, or prevent apoptosis, in definitive erythroid progenitors. However, we cannot completely rule out the indirect physiological influence (e.g. through hypoxia) that may result from the lack of primitive erythropoiesis. Gene rescue and/or selective inactivation experiments will be necessary to further refine the specific roles played by GATA-1 in definitive hematoipoiesis in vivo.

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Arrest in Primitive Erythroid Cell Development Caused by Promoter-specific Disruption of the GATA-1 Gene
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