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Mouse model of human β^0 thalassemia: Targeted deletion of the mouse β^{maj}- and β^{min}-globin genes in embryonic stem cells

(knockout mutation/hemoglobin A transgenic mice)

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ABSTRACT **β^0-Thalassemia is an inherited disorder characterized by the absence of β-globin polypeptides derived from the affected allele. The molecular basis for this deficiency is a mutation of the adult β-globin structural gene or cis regulatory elements that control β-globin gene expression. A mouse model of this disease would enable the testing of therapeutic regimens designed to correct the defect. Here we report a 16-kb deletion that includes both adult β-like globin genes, β^{maj} and β^{min}, in mouse embryonic stem cells. Heterozygous animals derived from the targeted cells are severely anemic with dramatically reduced hemoglobin levels, abnormal red cell morphology, splenomegaly, and markedly increased reticulocyte counts. Homozygous animals die *in utero*; however, heterozygous mice are fertile and transmit the deleted allele to progeny. The anemic phenotype is completely rescued in progeny derived from mating β^0-thalassemic animals with transgenic mice expressing high levels of human hemoglobin A. The β^0-thalassemic mice can be used to test genetic therapies for β^0-thalassemia and can be bred with transgenic mice expressing high levels of human hemoglobin HbS to produce an improved mouse model of sickle cell disease.**

Homozygous β^0-thalassemia in humans is characterized by severe anemia that begins during the first month of life (1). As the level of fetal hemoglobin ($\alpha_2\gamma_2$; HbF) declines, affected individuals are unable to produce the major adult hemoglobin ($\alpha_2\beta_2$; HbA) and become transfusion-dependent. Although transfusion therapy has improved survival in the past 10 years, significant complications from iron overload and hepatitis remain problematic. Several large studies reporting successful bone marrow transplantation have been published (2); however, the procedure is associated with high morbidity and some mortality, and fully matched donors are difficult to identify for many patients (2). Autologous bone marrow transplantation after transfer of a normal β-globin gene into hematopoietic stem cells is a major therapeutic goal but relatively inefficient transduction of stem cells and low levels of globin gene expression have hindered progress. The production of the animal model for β^0-thalassemia described in this paper may expedite the development of an effective gene therapy by providing a system to test improved gene transfer methods *in vivo*.

The mouse β-globin locus contains two embryonic genes, εy and βh1, and two fetal/adult genes, β^{maj} and β^{min} (Fig. 1*a*). Two mouse models of β-thalassemia have been reported previously; however, in both of these cases one of the two adult β-globin genes remained functional (3, 4). Detloff *et al.* (5) recently described an elegant method for deleting the β^{maj}- and β^{min}-globin genes in ES cells; however, mice derived from these cells have not been reported. In the present study, we describe a 16-kb deletion that includes both adult β-like globin genes, β^{maj} and β^{min}, in ES cells and the transmission of this mutation through the mouse germ line. Mice that are heterozygous for the deletion are viable but severely anemic.

MATERIALS AND METHODS

Targeting Vector Construction. Homologous sequences flanking β^{maj}- and β^{min}-globin genes were isolated from a 129 Sv/Ev strain mouse genomic library by using a 2.3-kb *Pst* I probe containing β^{maj}-globin gene sequences. The targeting vector was constructed by inserting a 1.7-kb *Hin*dIII fragment and a 7.0-kb *Bam*HI fragment into the *Hin*dIII and *Bam*HI sites of the plasmid pNTK (6, 7).

ES Cell Transfection and Characterization of Homologous Recombinant. The targeting vector was linearized with *Sal* I and introduced into the D3 line of ES cells as described (8). Briefly, 2×10^7 cells in 1 ml of Dulbecco's modified Eagle's medium with 15% (vol/vol) fetal calf serum (HyClone) were electroporated with 25 μg of linearized vector DNA in a 0.4-cm cuvette at 400 V and 250 μF with a Bio-Rad Gene Pulsar. Twenty-four hours after electroporation, cells were selected (9) in G418 (300 μg/ml) and 2.5 μM gancyclovir (Syntex, Palo Alto, CA) for 2 weeks. Forty colonies were picked and expanded, and DNA was isolated for Southern blot analysis. The 5′ probe was a 1.45-kb *Sau*3A–*Hin*dIII fragment and the 3′ probe was a 1.12-kb *Bam*HI–*Pst* I fragment (Fig. 1).

Characterization of Chimeras and Agouti Offspring. The probes used for Southern blot analysis of chimeras and agouti offspring were a 1.03-kb *Hin*dIII fragment from positions −340 to +690 of β^{maj}. This probe cross-hybridizes with β^{min}, β^s, and β^t sequences from the *Bam*HI site in the second exon to the end of this exon. Cellulose acetate gel electrophoresis was performed as described (10). Primer-extension analysis was performed as described (11). Primer-extension reaction mixtures contained 4 μg of RNA from 10-day yolk sac or 50 ng of RNA from adult blood. Bands were quantitated on a Molecular Dynamics PhosphorImager using IMAGEQUANT software.

RESULTS AND DISCUSSION

Fig. 1*b* illustrates the replacement vector that was designed to produce a 16-kb deletion encompassing both murine adult β-globin genes. Homologous recombination in ES cells between the replacement vector and the wild-type genome replaced β^{maj}- and β^{min}-globin genes with a selectable marker (Fig. 1*b*). Southern blot analysis with 5′ and 3′ DNA probes demonstrated a correctly targeted locus (Fig. 1 *b* and *c*). Cells with the mutated β-globin locus were injected into C57BL/6 blastocysts to generate 12 chimeric animals. Five of seven male

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Abbreviations: ES cell, embryonic stem cell; Hb, hemoglobin; LCR, locus control region.

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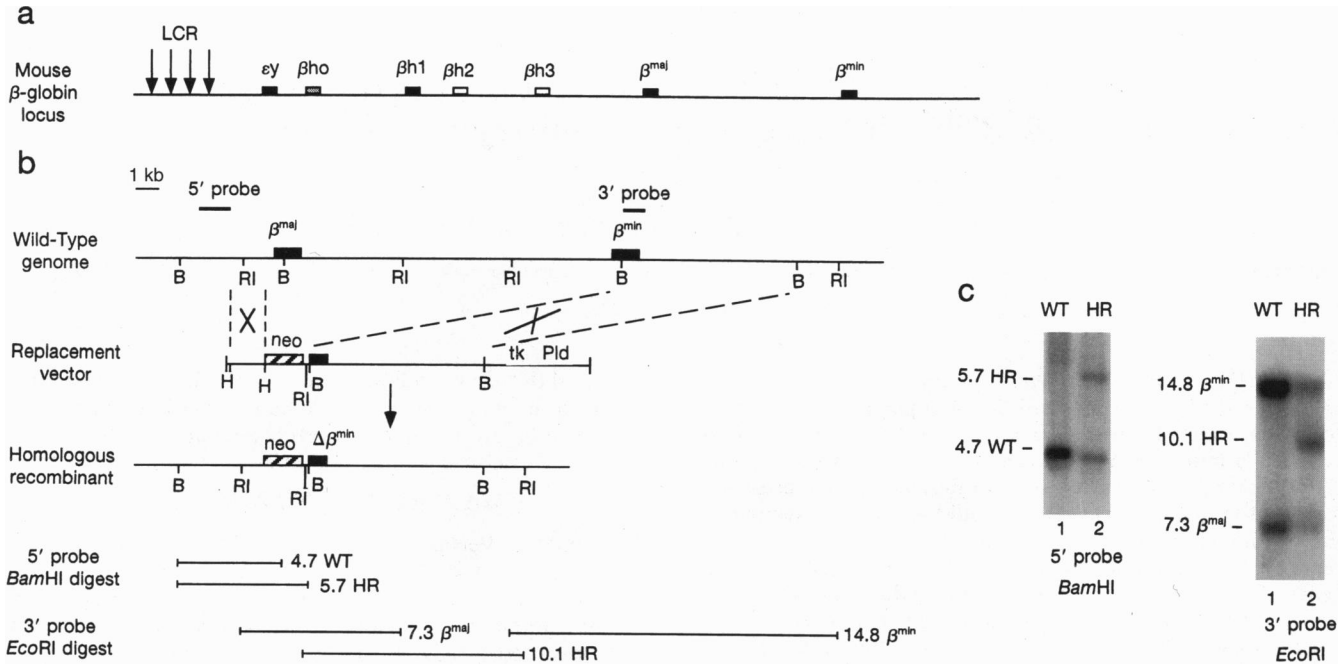

FIG. 1. Deletion of the adult β-globin genes in embryonic stem (ES) cells. (*a*) The mouse β-globin locus. The locus control region (LCR) is marked by four arrows indicating DNase I hypersensitive sites. The εy and βh1 genes are expressed in early embryonic development; the β^{maj} and β^{min} genes are expressed in adult erythroid tissue. βh0, βh2, and βh3 are pseudogenes. (*b*) Scheme for targeted deletion of the β^{maj}- and β^{min}-globin genes. The wild-type genome represents the arrangement of the β^{maj}- and β^{min}-globin genes separated by 15.2 kb on a normal allele. The replacement vector illustrates the targeting construct with 1.7 kb of 5′ homology and 7.0 kb of 3′ homology inserted into the pNTK vector (6, 7), which contains the neomycin resistance (neo) and herpes simplex virus thymidine kinase (tk) genes driven by the phosphoglycerate kinase (pgk) promoter. Pld, plasmid. Homologous recombinant illustrates the structure of the targeted chromosome. The entire β^{maj} gene is deleted and the β^{min} gene is deleted up to the *Bam*HI site in the second exon. The remaining lines illustrate the predicted *Bam*HI and *Eco*RI fragments that are generated in the correctly targeted locus. The 5′ probe is outside the targeting construct. The 3′ probe is a β^{min}-globin gene fragment that hybridizes to both β^{maj}- and β^{min}-globin sequences. (*c*) Southern blot analysis of DNA isolated from wild-type (lanes WT) D3 cells and from D3 cells containing the targeted deletion (homologous recombinant, HR).

chimeras produced agouti offspring when bred to C57BL/6 females.

Mice have several β-globin haplotypes; the two most common are Hbb^d and Hbb^s (Fig. 2*a*) (12–14). ES cells, which are derived from mouse strain 129, have the Hbb^d haplotype and, therefore, contain the β^{maj}- and β^{min}-globin genes. C57BL/6 mice have the Hbb^s haplotype; their genes are designated β^s- and β^t-globin. The newly targeted mutant locus is designated Hbb^o, because no functional adult β-globin gene is present. Restriction enzyme site polymorphisms in these haplotypes allow easy identification of the mutant allele in agouti offspring. Mice that inherit wild-type Hbb^d and Hbb^s alleles contain all four adult genes (Fig. 2 *a* and *b*, lane 3), whereas agouti animals that inherit the Hbb^o and wild-type Hbb^s alleles lack the β^{maj}- and β^{min}-globin genes (Fig. 2*a* and *b*, lane 4). Germ-line transmission of the deleted locus is also confirmed by cellulose acetate electrophoresis of denatured blood hemolysates from agouti offspring. All four β-globin polypeptide chains are present in agouti animals that inherit wild-type alleles from both parents (Hbb^d/Hbb^s; Fig. 2*c*, lane 3); however, no β^{maj}- and β^{min}-globin polypeptides are synthesized in animals that inherit the Hbb^o allele (Fig. 2*c*, lane 4).

Primer-extension analysis of adult blood RNA demonstrated a 25% reduction of steady-state β-globin mRNA levels in mutant agouti offspring compared to wild-type siblings (Fig. 3, lanes 3 and 4). A 50% reduction would be expected from deletion of the β^{maj}- and β^{min}-globin genes; however, up-regulation of the β^s- and β^t-globin genes on the wild-type chromosome partially compensates for the mutation.

In humans, β^0-thalassemias that result from complete deletions of the β-globin gene are usually accompanied by increased expression of the fetal γ-globin gene in cis to the affected allele (1) and these polypeptides partially compensate for the loss of β-globin chains. Enhanced γ-globin gene expression presumably results from interaction of fetal globin gene regulatory sequences with the upstream LCR. Although mice do not have a true fetal globin polypeptide, the mouse embryonic/fetal protein designated βh1 is 90% identical to the human γ-globin polypeptide. To determine whether the βh1 gene is up-regulated in mice with the 16-kb deletion, primer-extension analysis was performed on reticulocyte RNA (Fig. 3). Interestingly, no βh1-globin mRNA is observed in the Hbb^o/Hbb^s animals (Fig. 3, lane 4); similarly, no embryonic εy-globin gene expression is detected (data not shown; the εy polypeptide is 88% identical to the human γ-globin gene). The lack of εy and βh1 expression may result from active repression of the embryonic globin genes in adult erythroid tissue (15–18). Alternatively, the phosphoglycerate kinase promoter that is inserted in the mutant locus may form stable complexes with the LCR, excluding productive interactions with the embryonic gene promoters (4).

The morphology of red blood cells from Hbb^o/Hbb^s mice is similar to cells from homozygous human β^o-thalassemic patients. Erythrocytes from HR mice exhibit marked microcytosis and variation in size and shape (anisopoikilocytosis, Fig. 4*b*) compared to controls (Fig. 4*a*). Spiculated microcytes and hypochromic macrocytes are numerous and target cells are prevalent. The exaggerated central pallor of red cells reflects a significant reduction in cellular hemoglobinization. In control mice the Hb concentration is 14.9 ± 0.7 g/dl, while in mutant animals, Hb levels are only 7.1 ± 0.5 g/dl. Hbb^o/Hbb^s mice are also characterized by marked reticulocytosis (Fig. 4 *b* and *e*). Control animals have reticulocyte counts of 2.4 ± 0.5%, while reticulocyte levels in HR mice are elevated to 27.0 ± 1.6%. The bone marrow of Hbb^o/Hbb^s mice is hypercellular (Fig. 5*b*) compared to controls (Fig. 5*a*) and that the spleens

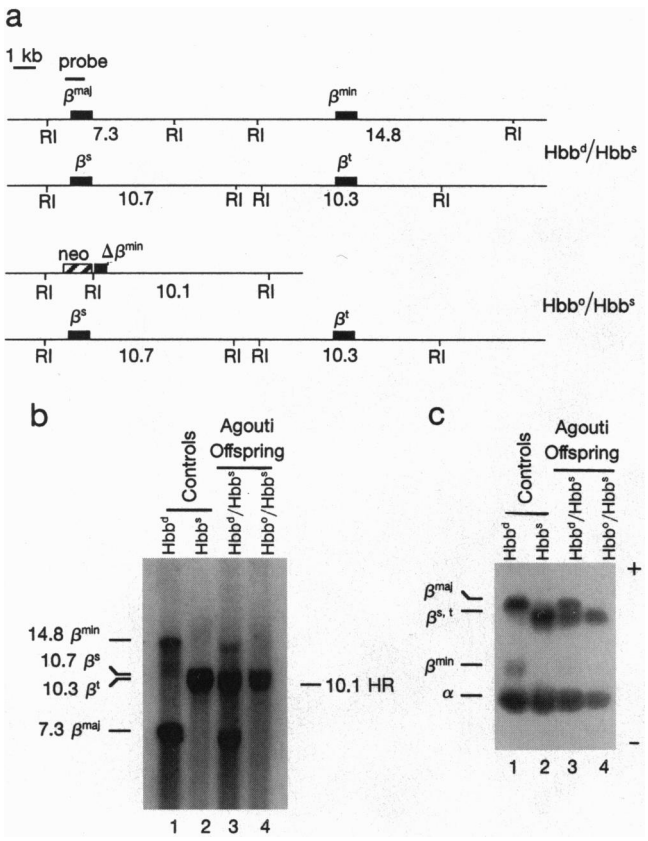

FIG. 2. Germ-line transmission of the β^{maj}/β^{min}-globin gene deletion. (*a*) Schematic representation of the β-globin locus in agouti offspring obtained from breeding chimeric males with C57BL/6 females. Agouti animals that inherit a wild-type allele from the chimera (β^{maj} and β^{min}; Hbb^d) and a wild-type allele from the C57BL/6 parent (β^s and β^t; Hbb^s) have the genotype shown at the top. Agouti animals that inherit a targeted allele from the chimera (Hbb^o) and a wild-type allele from the C57BL/6 parent (Hbb^s) have the genotype shown at the bottom. (*b*) Southern blot analysis of *Eco*RI-digested tail DNA from agouti offspring. The probe is a β^{maj}-globin fragment that hybridizes to β^{maj}-, β^{min}-, β^s-, and β^t-globin genes. Lanes 1 and 2 are controls for the Hbb^d and Hbb^s haplotypes, respectively; these are DNA from 129 and C57BL/6 mice. Lanes 3 and 4 are DNA of agouti litter mates from a cross of a chimeric male with a C57BL/6 female. In lane 3 the presence of all four β-globin gene fragments indicates that two wild-type alleles (Hbb^d and Hbb^s) were inherited. In lane 4 the absence of the β^{maj}- and β^{min}-globin gene fragments and the presence of the 10.1-kb band demonstrate that the deleted allele (Hbb^o) is inherited from the chimera. A wild-type allele (Hbb^s) containing β^s (10.7 kb) and β^t (10.3 kb) globin genes is inherited from the C57BL/6 female. (*c*) Cellulose acetate gel electrophoresis of denatured hemolysates from control and agouti offspring. Lanes 1–4 are hemolysates from the same agouti offspring analyzed in lanes 1–4 of *b*, respectively.

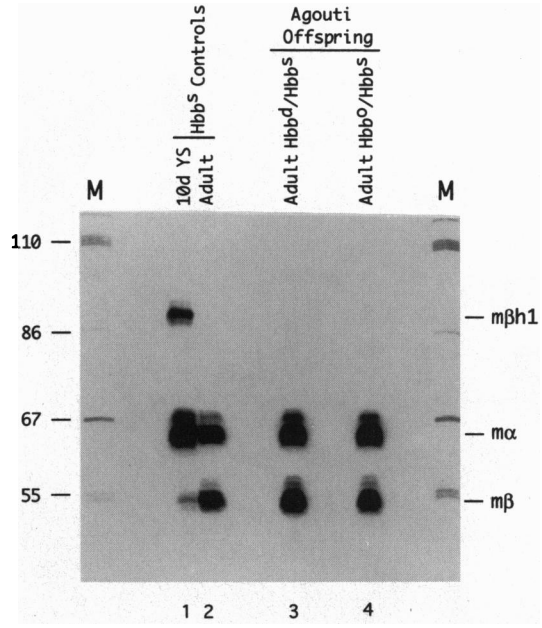

FIG. 3. Primer-extension analysis of globin gene expression. The relative levels of α-, β-, and βh1-globin mRNAs in adult reticulocytes were determined by primer extension. Lanes 1 and 2 are 10-day yolk sac and adult blood RNA controls from C57BL/6 mice. Adult blood RNA of agouti litter mates with the wild-type Hbb^d allele or the mutant Hbb^o allele are analyzed in lanes 3 and 4, respectively. The ratio of β-globin to α-globin mRNA is decreased 25% in the Hbb^o/Hbb^s mouse that is heterozygous for the β^{maj}/β^{min}-globin gene deletion (lane 4) compared to its sibling with four functional adult β-globin genes (lane 3). Note that embryonic βh1-globin gene expression is not up-regulated in Hbb^o/Hbb^s mutant mice (lane 4).

are dramatically enlarged (Fig. 5*c*). The average weight of spleens from Hbb^o/Hbb^s mice is 800 mg and the weight of control spleens is 80 mg.

The anemia of Hbb^o/Hbb^s mice is more severe than anemia in human patients who are heterozygous for β^0-thalassemia. Although the basis for this difference is unclear, the lack of εy and βh1 up-regulation in adult β^0-thalassemic mice may partially explain the discrepancy. As mentioned above, the human γ-globin gene is up-regulated in most β^0-thalassemias that result from complete deletions of the β-globin gene, and increased HbF levels may partially compensate for the decrease in HbA (1). Also free α-globin polypeptides may be more toxic in mice than in humans. When peripheral blood of Hbb^o/Hbb^s mice is stained with methyl violet, numerous inclusion bodies are observed in erythrocytes (Fig. 5*e*). These inclusion bodies, which presumably contain excess α-globin chains, are rare in wild-type animals (Fig. 5*d*). The precipitated globin polypeptides are more prevalent in the heterozygous β-thalassemic mice than in heterozygous human patients; in fact, the number of inclusion bodies in heterozygous mice is similar to homozygous human patients with severe β-thalassemia (19). These results suggest that free α-globin chains may be more toxic in mice than in humans. Perhaps proteases do not degrade free α-globin chains as efficiently in mice as in humans and/or α-globin polypeptides may interact more strongly with erythrocyte membranes in mice compared to humans. These speculations are testable and should be examined.

Although heterozygous, β^0-thalassemic mice are severely anemic, they are fertile and faithfully transmit the deleted allele to progeny. As expected, homozygous mutants produced by mating heterozygotes die *in utero* because of the complete absence of β-globin polypeptides. When heterozygous β^0-thalassemic animals are mated with transgenic mice that express high levels of human adult hemoglobin (HbA; 50% of total hemoglobin), the anemic phenotype is corrected in progeny that inherit both the Hbb^o allele and the human transgenes. Red blood cell morphology (Fig. 4*c*), hemoglobin concentration (14.6 g/dl), and reticulocyte counts (1.7%, Fig. 4*f*) are all normal in these animals. These results demonstrate that the anemic phenotype is completely rescued by expression of the human α- and β-globin transgenes. Homozygous β^0-thalassemic animals are also rescued by the human transgenes (data not shown).

The mice described in this report should be a useful animal model for the development of therapeutic treatments designed to alleviate human β-thalassemia, as well as other hemoglobinopathies. If the anemia in these thalassemic mice can be corrected by the introduction of normal β-globin genes into bone marrow stem cells, the experiments would provide a

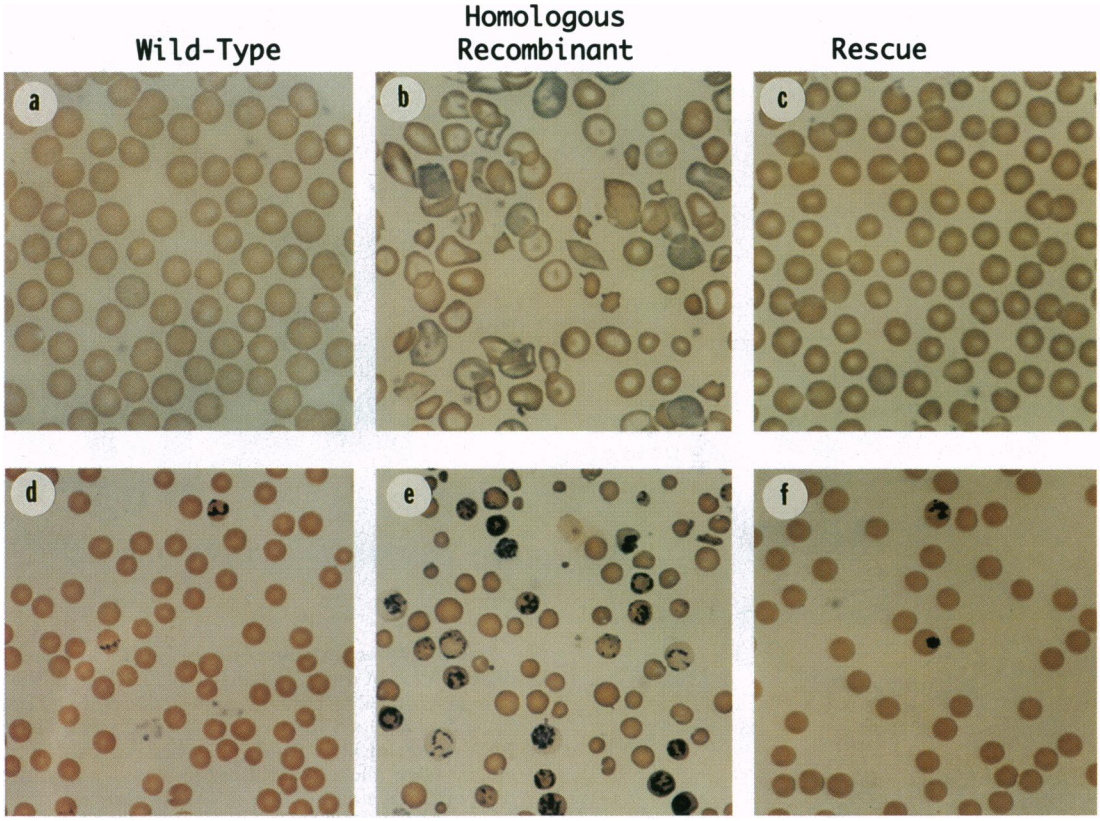

FIG. 4. Peripheral blood from wild-type, homologous recombinant, and rescued mice. (*a–c*) Peripheral blood stained with Wright stain. (*a*) Peripheral blood from a wild-type (Hbb^d/Hbb^s) agouti offspring demonstrates normal erythrocyte morphology. (*b*) Blood smear from a homologous recombinant (Hbb^o/Hbb^s) agouti offspring illustrates erythrocytes with severe anisopoikilocytosis. Numerous microcytic and hypochromic erythrocytes are visible as well as target cells. (*c*) Peripheral blood of progeny obtained from mating Hbb^o/Hbb^s mice with transgenic animals that synthesize high levels of human adult hemoglobin (HbA) (ref. 10 and T.M.R. and T.M.T., unpublished data). The abnormal phenotype was completely rescued. (*d–f*) Supravital reticulocyte staining with new methylene blue and counterstaining with Wright stain. (*d*) Reticulocyte stain of wild-type agouti offspring. (*e*) Reticulocyte stain of a homologous recombinant (Hbb^o/Hbb^s) agouti offspring. (*f*) Reticulocyte stain of progeny obtained from mating Hbb^o/Hbb^s mice with transgenic animals that synthesize high levels of human adult hemoglobin. Animals expressing high levels of HbA were produced by coinjection of LCR α and LCR γ–β transgenes (T.M.R. and T.M.T., unpublished data). These animals synthesize high levels of human HbF (50% of total hemoglobin) during early development and then switch to equally high levels of human HbA.

foundation for gene therapy in human patients with the disease. Recently, C. Paszty and E. Rubin (personal communication) deleted the mouse adult α-globin genes in ES cells and passed this mutation through the germ line. The appropriate matings between these α-thalassemic animals, Hbb^o/Hbb^s mice, and transgenic mice that synthesize high levels of human sickle hemoglobin (HbS) (20–26) should produce animals that synthesize HbS exclusively. If these animals display the *in vivo* pathology of sickle cell anemia as expected, they will serve as an excellent model of sickle cell disease. The animals may then be useful for developing therapeutic protocols for ameliorating or correcting the disease including the introduction of anti-sickling globin genes (27) into hematopoietic stem cells.

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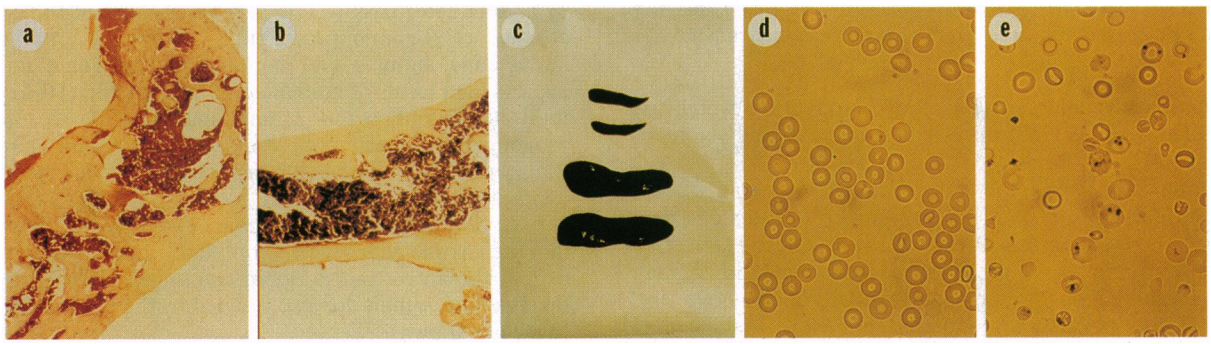

FIG. 5. Hypercellular bone marrow, splenomegaly, and inclusion bodies in thalassemic mice. (*a*) Bone marrow section of a wild-type animal. (*b*) Bone marrow section of an Hbb^o/Hbb^s animal illustrating hypercellularity. Both sections are stained with Giemsa. Numerous erythroid precursors are evident in higher magnifications (data not shown). (*c*) Spleens of wild-type (upper pair) and Hbb^o/Hbb^s (lower pair) animals. The spleens of thalassemic animals are enlarged 10-fold compared to controls. (*d* and *e*). Supravital stain of peripheral blood from wild-type (*d*) and Hbb^o/Hbb^s (*e*) animals with methyl violet. Numerous inclusion bodies, presumably containing free α-globin chains, are observed in Hbb^o/Hbb^s animals compared to controls.

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