

A mouse model for β^0 -thalassemia

(gene targeting/hemoglobin/splenomegaly/iron overload/extramedullary hematopoiesis)

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ABSTRACT We have used a “plug and socket” targeting technique to generate a mouse model of β^0 -thalassemia in which both the *b1* and *b2* adult globin genes have been deleted. Mice homozygous for this deletion (*Hbb*^{th-3}/*Hbb*^{th-3}) die perinatally, similar to the most severe form of Cooley anemia in humans. Mice heterozygous for the deletion appear normal, but their hematologic indices show characteristics typical of severe thalassemia, including dramatically decreased hematocrit, hemoglobin, red blood cell counts, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration, as well as dramatically increased reticulocyte counts, serum bilirubin concentrations, and red cell distribution widths. Tissue and organ damage typical of β -thalassemia, such as bone deformities and splenic enlargement due to increased hematopoiesis, are also seen in the heterozygous animals, as is spontaneous iron overload in the spleen, liver, and kidneys. The mice homozygous for the *b1* and *b2* deletions should be of great value in developing therapies for the treatment of thalassemias *in utero*. The heterozygous animals will be useful for studying the pathophysiology of thalassemias and have the potential of generating a model of sickle cell anemia when mated with appropriate transgenic animals.

The α - and β -globin polypeptide chains of hemoglobin are encoded by multigene clusters that are generally conserved throughout mammalian evolution. The mouse β -globin gene cluster on chromosome 7 (1) has four functional β -globin genes: *β H1*, an early embryonic globin gene; *$\epsilon\gamma^2$* , a late embryonic globin gene; and two adult globin genes, *b1* (β^{major}) and *b2* (β^{minor}) (2). Mouse embryonic hemoglobins are first expressed at 9.5 days of gestation in the yolk sac and later in fetal liver. The switch from predominantly embryonic to predominantly adult hemoglobins is completed between days 14 and 15 of gestation in the normal fetus (3, 4). The *b1* and *b2* genes are then expressed in fetal liver and spleen and, finally, in bone marrow during adult life. The *b1* gene is reported to be responsible for $\approx 80\%$ and *b2* for $\approx 20\%$ of adult β -globin production (5).

Human thalassemias are a heterogeneous group of inherited disorders of hemoglobin synthesis, all characterized by the absence of or reduction in amounts of one or more of the globin chains of hemoglobin (6), which generates an imbalance between the subunits of hemoglobin. When the reduction is among the adult β -globin genes, the resultant disorders are β -thalassemias. These disorders can be divided into two major groups, β^0 and β^+ , based on the quantity of β -globin produced; β^0 -thalassemia results from the complete absence of the β -globin, and β^+ -thalassemia results from a decrease in β -globin production.

No naturally occurring β^0 -thalassemia has been observed in mice, but two mouse models of β^+ -thalassemia exist (7, 8). In

the first, a spontaneous DNA deletion of ≈ 3.7 kb in the β -globin locus was found that includes the entire *b1* gene and its adjacent upstream sequences. Mice homozygous for the deletion (*Hbb*^{th-1}/*Hbb*^{th-1}) survive to adulthood in $\approx 60\%$ of the numbers with wild-type (wt) mice. The heterozygous mice exhibit a mild phenotype comparable to that of thalassemia trait in humans (7). The second model for β -thalassemia was created by targeted gene disruption of the mouse *b1* gene. Mice homozygous for an insertional disruption of the *b1* gene (*Hbb*^{th-2}/*Hbb*^{th-2}) are severely anemic and do not survive more than a few hours after birth (8). A possible explanation for the difference between the deletion and disruption mouse models of thalassemia is that the non-globin-producing promoters in the disrupted β -globin locus can compete for access to the locus control region needed for transcription, thereby reducing transcription from the normal *b2* gene (8). In the deletion animals, the *b2* gene has no competitors. Similar differences in transcription have been observed in human thalassemic patients with deletions or mutations in the promoter region of the adult β -globin gene compared to patients with deletions or mutations that do not affect the promoter region (9).

The mice homozygous for deletion of both *b1* and *b2* genes that we have generated in the present study have a severe form of β^0 -thalassemia, similar to the most severe forms of Cooley anemia in humans. The heterozygous animals show a pathophysiology comparable to that of patients with classical human β -thalassemia intermedia.

MATERIALS AND METHODS

Animals. All the wt mice and mice heterozygous for the β -globin gene deletion studied here are F₁ animals, except where stated otherwise. The F₁ animals are offspring from mating between chimeras and mice of strain C57BL/6J (strain B6) and they have a hybrid genetic background derived from the two inbred strains: B6 and 129Ola (strain 129). All mice were maintained in a room illuminated from 7 a.m. to 7 p.m. and were fed and watered *ad libitum*.

Gene Targeting. Deletion of the mouse *b1* and *b2* adult β -globin genes was accomplished by the two-step “plug and socket” gene targeting strategy as described (10). Three independently targeted embryonic stem (ES) cells were injected into host B6 blastocysts (11). Chimeric mice generated from two of them transmitted the targeted gene to their offspring. Mice heterozygous for the deletion have diagnostic bands of 13.0 or 12.4 kb when genomic DNA is digested with *Xba* I or *Hpa* I, Southern blotted, and probed with a 500-bp *HPRT* gene fragment containing the *HPRT* promoter and the first exon (10).

Collection and Hematologic Analysis of Blood. Blood samples were collected from the retroorbital sinus. For hemoglobin analysis, freshly drawn, heparinized blood samples were collected, and the red cells were washed once in 20 \times vol of phosphate-buffered saline and lysed in 5 \times vol of cystamine-

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Abbreviations: ES cells, embryonic stem cells; wt, wild type.

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containing buffer (12). Samples containing approximately equal amounts of hemoglobin were then run on cellulose acetate membranes (Helena Laboratories) (12). Hemoglobin bands were visualized by Ponceau S staining (Helena Laboratories).

For hematologic analyses, freshly drawn EDTA-treated blood was used. Peripheral blood smears were stained with Wright's stain (Sigma). Hematologic profiles were obtained with an automatic blood cell counter (Roche Cobas Helios Hematology Analyzer), which gives complete blood cell counts. Reticulocyte counts following supravital staining with Acoustain (1% new methylene blue/0.9% sodium chloride/1.6% potassium oxalate) (Sigma) were determined by standard methods (13). Serum bilirubin concentrations (total and direct) were determined on freshly collected blood using a reagent kit (procedure no. 605) from Sigma.

Scanning electron microscopy was performed on freshly drawn, heparinized blood samples. After washing once with saline, packed red blood cells were fixed with glutaraldehyde and allowed to settle on polylysine-coated glass coverslips. Blood cells were then dehydrated in a graded series of ethanol and critical-point dried. After drying, blood cells were coated with carbon followed by a 60:40 mixture of gold/palladium for viewing with a Cambridge scanning electron microscope at 10 kV.

Histology. Mice for histologic studies were sacrificed with a lethal dose of tribromoethanol (Avertin). The abdominal and thoracic cavities were opened and visceral organs were perfused through heart puncture with phosphate-buffered paraformaldehyde (4%; pH 7.4) under physiological pressure. Spleen, liver, heart, lungs, and kidneys were removed, weighed, embedded, and sectioned. Specimens were stained with hematoxylin and eosin and iron stains (Prussian blue).

Skulls and left femora from wt and heterozygous animals were removed, decalcified, embedded, and sectioned for histologic assessment by hematoxylin and eosin staining.

Statistical Analysis. The two independent samples *t* test was used (SYSTAT; see ref. 14) for comparing hematologic values between wt and heterozygous mice.

RESULTS AND DISCUSSION

Generation of β -Thalassemic Mice. In an earlier study from our laboratory, Detloff *et al.* (10) generated two targeted ES cell lines that had both the mouse adult β -globin genes (*b1* and *b2*) deleted. The deletion plug $\beta\Delta$ plug1.5 was also reinserted into the socket-containing ES cell line B20 as described (10), and Southern blot analysis revealed the presence of the desired deletion in one of six hypoxanthine/aminopterin/thymidine-resistant colonies. The three targeted ES cell lines were used to generate chimeric animals by injection into host blastocysts. Of 10 chimeras generated from two of the three targeted ES cell lines, two males and two females transmitted the ES cell genome to their offspring after being mated to mice of strain B6. The resulting (B6 \times 129) F_1 hybrids were initially genotyped by Southern blot analysis for the *b1* and *b2* globin gene deletion using genomic DNA from tail biopsies. Subsequently, heterozygotes were genotyped by blood smears, since their smears were so different from those of wt mice.

Body Weight Difference. The F_1 heterozygotes at the time of birth are pale and small in comparison with their wt littermates. At weaning (3 weeks), significant differences ($P < 0.005$) in body weight between F_1 heterozygotes and male or female F_1 wt animals were observed (Table 1). Body weight differences between wt F_2 and heterozygous F_2 pups at weaning are smaller although still significant ($P < 0.05$, if all mice, both male and female, are compared; see Table 1). Body weight differences in the F_1 mice decreased with age, becoming statistically insignificant by 8–10 weeks of age (data not shown).

Homozygous Animals. Eight intercrosses between F_1 heterozygotes yielded 45 pups and 5 stillborn animals. Seven live-born pups were extremely pale and died within 12 hr. All 7 mice dying within 12 hr and the 5 stillborn ones were determined to be homozygous for the *b1* and *b2* deletions. The overall numbers of each genotype were not statistically different from Mendelian expectations (15 wt, 23 heterozygotes, and 12 homozygotes; $P \approx 0.7$; χ^2 analysis). The mice homozygous for the *b1* and *b2* deletions resemble human patients with the transfusion-dependent homozygous forms of β^0 -thalassemia that are lethal in the untreated state. They differ from humans in that they die within hours after birth rather than later in childhood. The increased severity of the phenotype in mice probably occurs because the genetic switch from embryonic to adult hemoglobin occurs 7–8 days before birth in mice. In humans, the switch from synthesizing fetal to adult hemoglobin is not completed until several months to a year after birth, and untransfused human β^0 -thalassemia homozygotes die in late infancy (15).

Red Cell Morphology. Blood smears (data not shown) and scanning electron micrographs (Fig. 1) were obtained using the blood of wt and heterozygous mice 7 weeks of age. The heterozygotes show characteristics typical of those seen in patients with severe thalassemia. There is marked anisocytosis and poikilocytosis; there are many microcytes, occasional polychromatic macrocytes, and frequent target cells. The red cells are generally hypochromic with variable numbers of teardrop and oval forms, and fragmented cells are seen. Nucleated red blood cells are also present. Pappenheimer bodies from precipitated α -globin chains are seen in the red blood cells.

Hematologic Studies. Hematologic studies included measurements of total hemoglobin, red blood cell count, hematocrit, reticulocyte count, and serum bilirubin concentrations (total and indirect) on wt and heterozygous mice 6–7 weeks of age. A summary of the results is shown in Table 2. Mice heterozygous for the deletion are anemic, as is reflected in the markedly decreased hematocrit, hemoglobin concentration, and red blood cell count. Their reticulocyte counts are dramatically increased (21-fold), indicating a state of active erythropoiesis in the heterozygous animals. The elevated serum concentrations of total and indirect bilirubin in heterozygous animals indicate an increased rate of red blood cell turnover and hemolysis. The high reticulocyte counts, low red blood cell counts, and increased indirect bilirubin in heterozygous mice indicate that both production and destruction of erythrocytes are increased in heterozygous compared to wt mice.

Table 1. Body weight of weanlings

	F ₁ cross			F ₂ cross		
	All mice	Male	Female	All mice	Male	Female
wt	11.8 \pm 0.4 (<i>n</i> = 24)	11.7 \pm 0.6 (<i>n</i> = 14)	12.0 \pm 0.4 (<i>n</i> = 10)	9.2 \pm 0.3 (<i>n</i> = 47)	9.1 \pm 0.3 (<i>n</i> = 31)	10.1 \pm 0.5 (<i>n</i> = 16)
Heterozygotes	9.0 \pm 0.6 (<i>n</i> = 18)	8.7 \pm 0.7 (<i>n</i> = 10)	9.4 \pm 0.6 (<i>n</i> = 8)	8.5 \pm 0.2 (<i>n</i> = 39)	8.5 \pm 0.3 (<i>n</i> = 23)	8.4 \pm 0.3 (<i>n</i> = 16)
<i>P</i>	0.00001	0.003	0.001	0.044	0.218	0.009

Body weights are expressed as means \pm SEM.

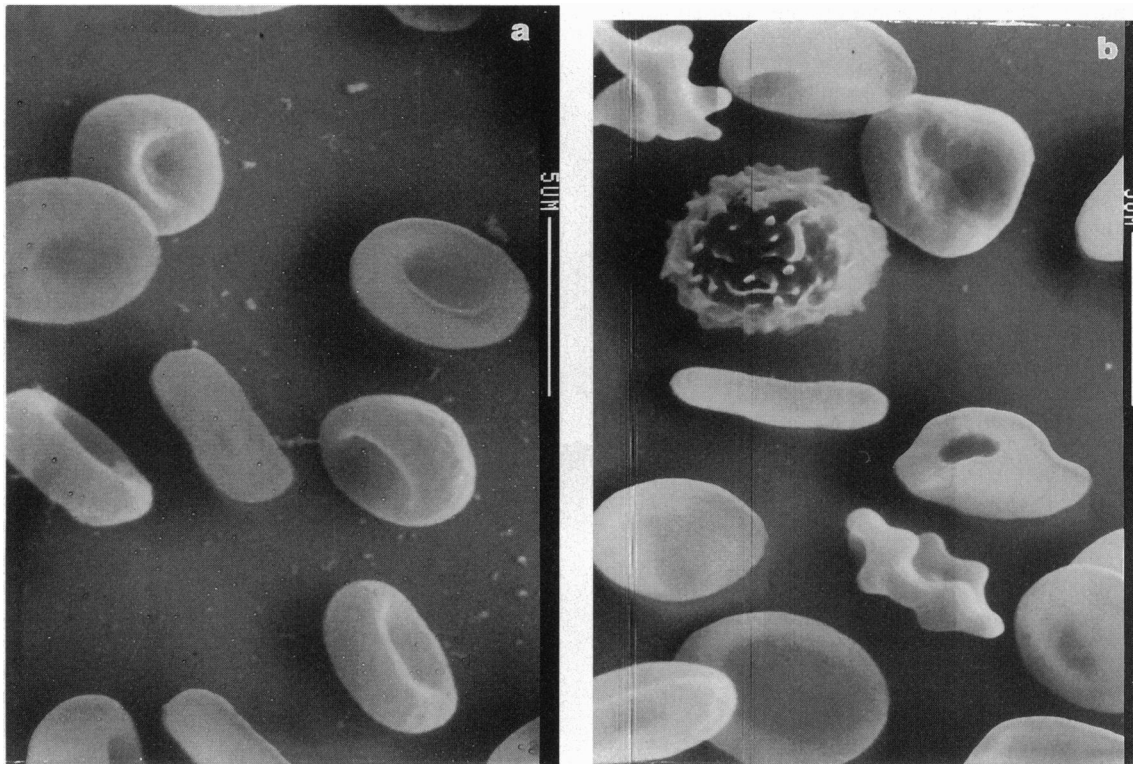


FIG. 1. Scanning electron micrograph of blood cells from a heterozygous F₁ β -thalassemic mouse and a wt littermate at 7 weeks of age. (A) wt mouse. (B) Mouse heterozygous for *b1* and *b2* deletions. ($\times 5390$.)

Human thalassemic patients have a characteristic hypochromic, microcytic anemia, with decreased mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration and increased red cell distribution width (15). Mice heterozygous for the *b1* and *b2* gene deletions have similar changes (Table 2) in all these parameters.

Hemoglobin Electrophoresis. The absence of β -globin production from the altered strain 129-derived β -globin locus was established by studying animals heterozygous for a normal β -globin locus that directs synthesis of a distinguishable form of adult β -globin. Hemoglobin from F₁ heterozygous animals have a wt (*Hbb^s* haplotype) chromosome from B6 and the strain 129-derived deletion chromosome, which would normally direct the synthesis of major and minor β -globin (*Hbb^d* haplotype). When cystamine-modified hemoglobins are separated by cellulose acetate electrophoresis (12), wt (B6 \times 129)F₁ mice have the B6-derived single and the 129-derived major and minor β -globin (Fig. 2, lane 2). The heterozygous thalassemic mice have only the B6-derived single β -globin (lane 3), verifying that the deletion chromosome contributed no adult β -globin chains (5).

Pathology and Histology. Pathologic changes revealed by gross and microscopic examination of the heterozygous animals were similar to those of human patients with severe

thalassemia. Spleen, liver, heart, lung, and kidney weights were determined as ratios to total body weight for both wt and heterozygous mutant animals. There were no differences in these ratios for liver, heart, lungs, and kidneys between wt and heterozygous animals. There was, however, a highly significant increase ($P < 0.001$) in the spleen weight/body weight ratio from heterozygous animals compared to wt control mice (2.80 ± 0.24 mg/g for wt mice versus 21.61 ± 4.54 mg/g for thalassemic mice). Splenomegaly is a common feature in human thalassemia patients.

Histologic studies of the heterozygotes also revealed significant changes. Again the most dramatic changes appeared to be in the spleens of the animals due to increased extramedullary hematopoiesis (Fig. 3). Spleen sections of wt mice show the typical normal histology with distinct red and white pulp in approximately equal proportions, with the white pulp-containing follicles having germinal centers and the red pulp having hematopoietic cells. Megakaryocytes are present in the red pulp, and trabeculae can be seen. In the spleens from mice heterozygous for the *b1* and *b2* deletions, there is no clear distinction between red and white pulp. Instead, the white pulp is almost completely replaced by hematopoietic tissue, and there is impressive erythroid hyperplasia. Occasional germinal centers can be seen perivascularly. More megakaryocytes are

Table 2. Hematologic values

	Hct, %	Hb, g/dl	RBC, $10^6/\text{mm}^3$		MCV, μm^3	MCH, pg	MCHC, g/dl	RDW	Bilirubin, mg/dl	
			Retics, %						Total	Indirect
wt F ₁	45.7 \pm 0.8 (n = 30)	16.5 \pm 0.5 (n = 28)	9.5 \pm 0.4 (n = 27)	1.4 \pm 0.4 (n = 5)	51.1 \pm 0.2 (n = 18)	18.6 \pm 0.2 (n = 18)	365 \pm 0.4 (n = 18)	17.4 \pm 0.6 (n = 18)	0.18 \pm 0.07 (n = 10)	0.14 \pm 0.03 (n = 10)
Heterozygous F ₁	30.5 \pm 0.8 (n = 28)	10.9 \pm 0.3 (n = 28)	6.6 \pm 0.1 (n = 24)	30.3 \pm 0.9 (n = 4)	43.0 \pm 0.5 (n = 17)	17.6 \pm 0.4 (n = 17)	30.7 \pm 0.4 (n = 17)	46.1 \pm 0.5 (n = 17)	0.45 \pm 0.06 (n = 7)	0.36 \pm 0.07 (n = 7)
<i>P</i>	<0.00001	<0.00001	<0.00001	<0.00001	<0.00001	0.014	<0.00001	<0.00001	0.00004	0.00004
% changes	-33	-34	-31	2064	-16	-5	-16	165	150	157

Hematologic values are expressed as means \pm SEM. Hct, hematocrit; RBC, red blood cells; Retics, reticulocytes; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, MCH concentration; RDW, red cell distribution width.

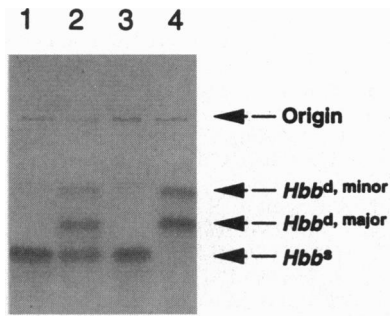


FIG. 2. Cellulose acetate electrophoretic analyses of mouse hemoglobins. Cystamine-modified hemoglobin was electrophoresed on cellulose acetate gel. Lanes 1–4, hemoglobins from wt mice of strain B6, wt F₁ mice, F₁ mice heterozygous for *b1* and *b2* deletions, and wt mice of strain 129, respectively.

also present. Iron stains of spleen sections from wt mice reveal only trace amounts of iron in macrophages, while the thalassemic mice have abundant, heavily stained iron particles in what appear to be “nurse cells,” the macrophages in the center of an area of erythropoiesis.

In both the bone and bone marrow of skulls and femora, significant differences were seen between wt and heterozygous mice. wt mice have thicker cortical bone in their skulls than do age-matched thalassemic mice. The femora of wt mice also have a shorter zone of proliferation and a longer zone of osteogenesis compared to those from thalassemic mice; this may contribute to the growth retardation seen in thalassemic mice. Relative to wt, mice heterozygous for the deletion have increased bone marrow cellularity as well as increased numbers of megakaryocytes and erythroid precursors.

In the older animals (5–6 months), histologic studies of the heterozygous and wt animals show that the spleen is still the major affected organ. The histologic differences are similar to those seen in younger animals, except that splenic iron deposits are significantly increased in the thalassemic mice, and iron deposits are now observed in both red and white pulp. The thalassemic mice have abnormal iron accumulation in the proximal convoluted tubules of their kidneys. This is not seen in wt mice and is indicative of hemolysis comparable to that seen in human thalassemic patients. Liver iron stores/deposits, which are minimal in wt animals, are abundant in both the hepatocytes and Kupffer cells of thalassemic animals (Fig. 4).

Iron Deposits. Our study provides clear evidence of spontaneous iron deposition in the β -thalassemic mice. Spontaneous iron overload in nontransfused human thalassemia patients arises not from an intrinsically flawed iron regulation or from an excess of dietary iron but rather from a response to the abnormal stimulation of increased erythropoietic activity (16–18).

Iron deposition is evident in mice heterozygous for the *b1* and *b2* deletions, even at a young age (4–5 weeks), in both livers and spleens. Iron accumulation in these organs was

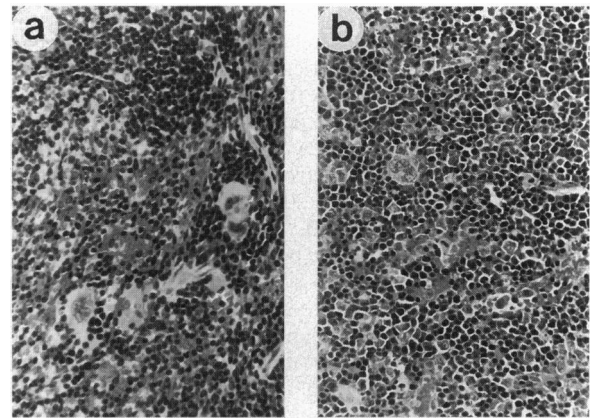


FIG. 3. Histology of spleens from a heterozygous F₁ β -thalassemic mouse and a wt littermate. Hematoxylin and eosin staining of spleen from a 6-week-old wt mouse (a) and a 6-week-old mouse heterozygous for the *b1* and *b2* deletions (b). ($\times 100$)

progressive. Garrick *et al.* (17) observed that iron deposition increases with age until plateaus in iron density and total iron content are reached at 200 days of age in Hbb^{th-1} homozygotes. Raja *et al.* (18) found that liver iron deposition increases in Hbb^{th-1} homozygotes until the age of 7–8 weeks, but it shows no further increase over successive weeks.

Hemosiderosis is the result of translocation of iron from the hemoglobin of red cells into the cells of the reticuloendothelial system in liver, spleen, and bone marrow. Normally, siderosis is innocuous and reversible and is even seen as a seasonal phenomenon in some animal species (19). However, continuous accumulation of iron in liver parenchymal cells can have deleterious effects. Although in humans iron overload occurs mainly in hypertransfused thalassemic patients, it can occur in young thalassemic patients before any transfusional therapy (20, 21). In these individuals, iron particles are mainly seen in periportal liver parenchymal cells.

Comparison of Mouse Models of β -Thalassemia. Mice heterozygous for the *b1* and *b2* deletions (which we propose to designate Hbb^{th-3}) have similar, although more severe, abnormalities in their hematologic parameters and pathologic and histologic findings to those of mice homozygous for the Hbb^{th-1} mutation (7). Mice homozygous for insertional disruption of the *b1* gene (Hbb^{th-2}/Hbb^{th-2}) have a more severe phenotype than that of the heterozygous Hbb^{th-3}/Hbb^s mice or than that of Hbb^{th-1}/Hbb^{th-1} mice homozygous for the *b1* deletion (8). The relative severities of the three conditions are of some interest. The severity of β -thalassemia is directly related to the imbalance in synthesis between α - and β -globins and so to the excess amount of α -globin produced (22). In normal strain 129 mice, there are two *b1* and two *b2* genes; the two *b2* genes produce $\approx 20\%$ of all β -globin chains (5). In the absence of any compensatory and competitive effects, mice homozygous for the Hbb^{th-1} *b1* deletion or the Hbb^{th-2} *b1* disruption would be expected therefore to have only $\approx 20\%$ of normal β -globin

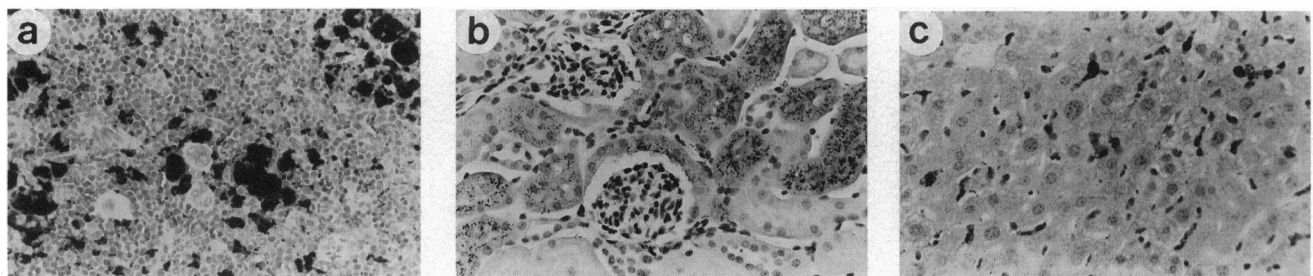


FIG. 4. Histology of spleen, kidney, and liver from F₁ heterozygous β -thalassemic mice. Iron staining of spleen (a), kidney (b), and liver (c) from a 5-month-old F₁ mouse heterozygous for the *b1* and *b2* deletions. Dark stained particles within cells are iron deposits. ($\times 400$)

production, while mice heterozygous for the *Hbb*^{th-3} *b1* and *b2* deletions would be expected to have ≈50% of normal β-globin synthesis. However, in practice mice homozygous for the *Hbb*^{th-1} mutation (which have only the two adult *b2* genes) produce more β-globin than expected (≈75% of normal rather than the expected 20%) (23), while mice homozygous for the *Hbb*^{th-2} mutation do not survive and probably produce less β-globin than the expected 20% (B. M. Brunner-Jackson, W. R. Shehee, O.S., and J. B. Whitney, unpublished data). These altered expressions have been attributed to translational competition between α- and β-globin mRNAs and/or transcriptional competition for access to the locus control region among all promoters (globin and nonglobin) in the β-globin locus (8, 24). In *Hbb*^{th-1}/*Hbb*^{th-1} mice, the *b2* gene promoter has no competitor, while in *Hbb*^{th-2}/*Hbb*^{th-2} mice the *b2* gene promoter has both the *neo* gene promoter and the nonproductive *b1* gene promoter as competitors. In our *Hbb*^{th-3} mice, with the deletion of both *b1* and *b2* genes, any compensatory and/or competitive factors within the β-globin gene locus are eliminated.

CONCLUSION

Mice heterozygous for the *Hbb*^{th-3} deletion generated in this study have a similar yet more severe phenotype than mice homozygous for the *Hbb*^{th-1} deletion or heterozygous for the *Hbb*^{th-2} insertion. The *Hbb*^{th-3} heterozygotes have anemia, splenomegaly, and bone abnormalities similar to those of human β⁰-thalassemic heterozygotes. However, unlike most untransfused human thalassemics, the animals develop spontaneous iron deposits very early. The animals should consequently be useful not only for studying the pathophysiology of thalassemias but also for studying iron overload in different organs. They have the potential of being useful for testing new ways of treating these diseases and the accompanying iron overload. In addition, the heterozygotes will be useful for mating with appropriate transgenic animals to generate a potentially improved mouse model of sickle cell anemia. Mice homozygous for the *Hbb*^{th-3} deletion may be valuable in developing *in utero* therapies for the treatment of severe and lethal forms of thalassemia.

Note Added in Proof. After submission of this manuscript, a report describing similar work was published (25).

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