Knockout-Transgenic Mouse Model of Sickle Cell Disease

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When transgenic mice that expressed human sickle hemoglobin were mated with mice having knockout mutations of the mouse α- and β-globin genes, animals were produced that synthesized only human hemoglobin in adult red blood cells. Similar to many human patients with sickle cell disease, the mice developed a severe hemolytic anemia and extensive organ pathology. Numerous sickled erythrocytes were observed in peripheral blood. Although chronically anemic, most animals survived for 2 to 9 months and were fertile. Drug and genetic therapies can now be tested in this mouse model of sickle cell disease.

The molecular basis for sickle cell disease is an A to T transversion in the sixth codon of the human β-globin gene (*1*). Accumulation of fibers containing long polymers of hemoglobin S (HbS) reduces the flexibility of red blood cells and leads to the occlusion of small capillaries (*2*). Intracellular fiber formation also results in erythrocyte membrane damage and increased red cell lysis (*3*). The ensuing disease is characterized by a chronic hemolytic anemia with episodes of severe pain and tissue damage that often result in kidney failure, liver pathology, stroke, infection due to splenic infarction, and other complications.

The development of a mouse model of the disease would permit the testing of novel drugs to alleviate symptoms and genetic therapies designed to correct the defect. The first step toward this goal was accomplished when Ryan *et al.* (*4*) and Greaves *et al.* (*5*) produced transgenic mice that synthesized about 50% human sickle hemoglobin and 50% mouse hemoglobin. However, these animals mimicked the sickle trait rather than sickle cell disease. Subsequently, other mouse models were produced (*6*). Although all of these mice provided important insights, none of them modeled the severe hemolytic anemia observed in human sickle cell disease. The absence of severe anemia most likely resulted from expression of endogenous murine hemoglobins in adult erythroid cells (*7*). To overcome this problem, Ciavatta *et al.* (*8*) and Yang *et al.* (*9*) deleted both of the adult mouse β-globin genes in embryonic stem cells and Paszty *et al.* (*10*) deleted both adult mouse α-globin genes. Mice derived from these cells not only provided valuable models of human β- and α-thalassemia (*8–10*) but also generated genetic backgrounds for the production of mice that synthesize only human hemoglobins in adult red blood cells as described below.

Sickle cell disease is a relatively benign disorder in the first few months of life because of the potent antisickling properties of human fetal hemoglobin (HbF) (*11, 12*). HbF, which constitutes 70 to 90% of total hemoglobin at birth, is gradually replaced by HbS during the first few months of life. Rising HbS concentrations result in the onset of disease between 3 and 6 months of age. A comprehensive mouse model of sickle cell disease would mimic the temporal switch of hemoglobins in humans. Animals containing cosmid and yeast artificial chromosome (YAC) transgenes (*13*) complete the switch from human γ-globin to β-globin gene expression by 15 days of gestation (6 days before birth). As a means to circumvent potential perinatal lethality resulting from an early switch to HbS, DNA constructs designed to delay hemoglobin switching to approximate the fetal to adult globin gene switch in humans were produced and tested in mice (*14*).

A 22-kb DNA fragment encompassing the human β-globin locus control region (LCR) was linked to a 9.7-kb DNA fragment containing the $^{A}\gamma$-globin and β^{S}-globin genes (Fig. 1A) (*15*). The LCR was also linked to a 3.8-kb DNA fragment containing the human α1-globin gene (*4*). Six independent lines of transgenic mice were produced by coinjecting these constructs, and the animals were subsequently bred to the mouse α- and β-globin knockout lines (*8, 10*). Mice heterozygous for the transgenes and heterozygous for both knockout loci were interbred to produce transgenic animals that are homozygous for the knockout alleles (*16*).

Adult HbS transgenic animals that are homozygous for both of the knockout alleles (HbS $m\alpha^{0/0}$ $m\beta^{0/0}$) synthesize no murine hemoglobin. In high-performance liquid chromatography (HPLC) profiles, only human α-, $^{A}\gamma$-, and β^{S}-globin chains were observed in LCR α/LCR $^{A}\gamma$-β^{S} transgenic mice that are doubly homozygous for the murine α- and β-globin gene knockout alleles (HbS3 $m\alpha^{0/0}$ $m\beta^{0/0}$) (Fig. 1B). The mice express high concentrations of HbF in the yolk sac blood islands early in development (8.5 days) and initiate a switch to HbS when the site of hematopoiesis shifts to the fetal liver (*17*). High concentrations of HbF (30 to 50%) persist in newborn HbS mice but gradually decline as the switch to HbS is completed at about 1 month of age. In adult mice, the mean values of $\gamma/\gamma+\beta^{S}$ for HbS lines 1 through 6 are 3.2 to 7.7% (Table 1). The average HbF concentrations in homozygous sickle cell (HbSS) patients is 5.8% with a range between 0.4 and 18.8% (*11, 12, 18*).

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Table 1. Hematological parameters, human γ chain levels, globin chain synthesis ratios, and urine osmolality of HbS mice. The RBC counts, hemoglobin concentrations, PCVs, and reticulocyte counts were measured for control animals and six HbS lines (*23, 24*). Human γ chain levels were calculated from the HPLC profiles of hemolysates and expressed as the percentage of total γ plus β^{s} chains (*22*). Globin chain synthesis ratios (α/non-α) and urine osmolality were determined for line HbS3 and control mice (*19, 21*). The individual values (n = 1 or 2) or the mean value ± SD ($n \geq 3$) for each measurement is listed for each group of animals. NA, not applicable; ND, not determined.

Line	RBC (×10^6/μl)	Hb (g/dl)	PCV (%)*	Reticulocytes (%)	γ/γ + β^{S} (%)	Globin chain syn. (α/non-α)	Urine osmolality (mOsm)
Control	8.5 ± 0.9 (n=7)	15.0 ± 0.8 (n=7)	46 ± 2.6 (n=7)	3.9 ± 1.1 (n=7)	NA	1.00 ± 0.02 (n=4)	1541 ± 360 (n=6)
HbS1	3.6 ± 0.7 (n=3}	4.1 ± 0.8 (n=3)	24 ± 3.8 (n=3)	56 ± 16 (n=8)	3.2 ± 0.6 (n=11)	ND	ND
HbS2	2.5 ± 0.8 (n=3)	4.5 ± 0.9 (n=3)	18 ± 4.5 (n=3)	68 ± 5 (n=5)	3.9 ± 1.9 (n=9)	ND	ND
HbS3	3.3 ± 1.0 (n=5)	6.1 ± 1.2 (n=5)	22 ± 4.7 (n=5)	57 ± 16 (n=5)	7.7 ± 2.2 (n=13)	1.15 ± 0.01 (n=3)	807 ± 285 (n=7)
HbS4	1.8 and 4.4	3.2 and 7.9	13 and 30	77 and 36	6.7 ± 0.6 (n=3)	ND	ND
HbS5	2.4 and 3.8	5.2 and 7.5	20 and 29	82 and 44	6.0 ± 1.7 (n=9)	ND	ND
Hbs6	5.5	5.1	27	47	3.4	ND	ND

*Mean cell volume is not listed but can be calculated by PCV/RBC × 10.

Globin chain synthesis was performed to assess the balance of α- and non–α-globin chains in adult mice (Table 1) (*19*). In humans with sickle cell disease, an α to non-α ratio of 0.9 to 1.1 is normal; ratios of 0.8 to 0.5 and 1.2 to 2.0 are indicative of mild to severe α- and β-thalassemia, respectively (*20*). In control mice the alpha to non-alpha ratio was 1.00 ± 0.02 (mean ± SD), and in HbS3 mice the ratio was 1.15 ± 0.01. Therefore, globin chains in HbS3 mice are relatively balanced, although some individuals are mildly β-thalassemic.

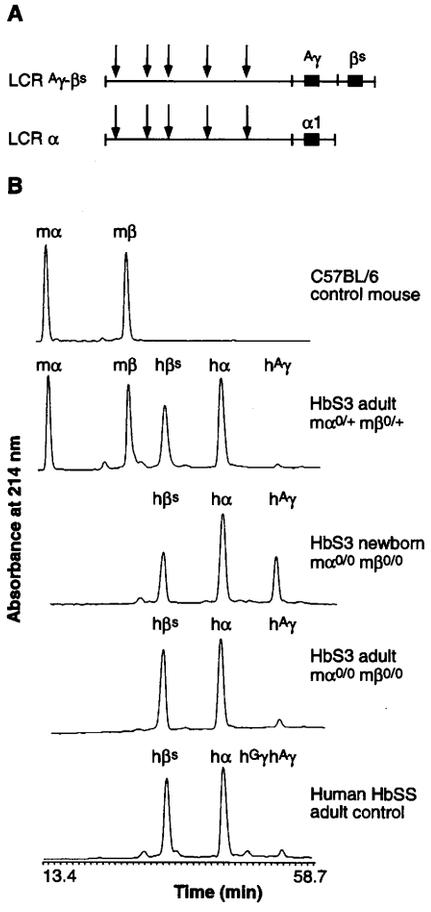

Fig. 1. DNA constructs and HPLC profiles. (**A**) DNA constructs used to produce transgenic mice (*15*). (**B**) Reverse-phase HPLC of adult and newborn hemolysates. HPLC profiles (*22*) of hemolysates from an adult HbS3 double heterozygote ($m\alpha^{0/+}$ $m\beta^{0/+}$), a newborn HbS3 double homozygote ($m\alpha^{0/0}$ $m\beta^{0/0}$), and an adult HbS3 double homozygote ($m\alpha^{0/0}$ $m\beta^{0/0}$) demonstrate the globin chains synthesized by these mice. C57BL/6 and human HbSS are mouse and human controls, respectively. LCR α/LCR $^{A}\gamma$-β^{S} transgenic mice that are doubly homozygous for the murine α- and β-globin gene knockout alleles produced only human α-, γ-, and β^{S}-globin chains as newborns and adults. Quantitation of γ and β^{S} chains in the HbS3 newborn sample demonstrated that γ polypeptides ($^{A}\gamma$ only) are 42% of the total γ + β^{S} chains. In the adult HbS3 mouse hemolysate, γ polypeptides ($^{A}\gamma$ only) were 6.7% of the total γ + β^{S} chains, which is similar to 8.9% ($^{A}\gamma$ + $^{G}\gamma$) calculated for the human HbSS sample.

Knockout-transgenic animals develop a severe hemolytic anemia. All HbS animals have a marked reduction in red blood cell (RBC) counts, hemoglobin (Hb) concentrations, and packed cell volumes (PCVs) and have significantly increased reticulocyte counts compared with control mice (Table 1). Numerous sickled cells were observed in peripheral blood smears of all the HbS lines as exhibited in the HbS3 sample (Fig. 2). Similar to human HbSS patients (Fig. 2), anisocytosis and poikilocytosis are evident, and target cells and nucleated RBCs are observed. Peripheral blood smears from HbS mice have a marked polychromasia indicative of large numbers of reticulocytes commensurate with increased erythropoiesis. This reticulocytosis results from severe anemia and is consistent with the marked expansion of erythropoiesis observed in the spleen of these mice. Because the spleen is a major hematopoietic tissue in anemic mice, HbS animals attempt to compensate for severe hemolysis by increased erythropoiesis in their spleens. Individual spleens in these animals ranged from 7 to 20 times the mass of normal nonanemic mouse spleens. The spleens of some HbS animals were 10% of the animal's body weight. The hemolytic anemia described above develops after the first few weeks of life as the concentration of HbF declines in these mice; this temporal pattern mimics the onset of anemia in human sickle cell infants during the first few months of life.

Unlike earlier mouse models of sickle cell disease, knockout-transgenic HbS mice develop significant in vivo pathology at a relatively young age under ambient conditions (Fig. 3A). As mentioned previously, the spleens of sickle cell mice are massively enlarged. The normal splenic architecture of red and white pulp is obliterated in the HbS mice by the large expansion of erythroid precursors. The red pulp (up to 95% of some spleens) comprised about equal numbers of pooled sickled RBCs and erythroid progenitors. Under higher magnification, dense mats of sickled erythrocytes were clearly observed in the splenic sinusoids. Electron micrographs of these cells demonstrated intracellular, human HbS fibers under normal oxygen tensions (Fig. 3B). The extremely low RBC counts and Hb concentrations of sickle cell mice can be explained by the destruction of sickled erythrocytes in the peripheral circulation and by the large numbers of RBCs that are continually trapped inside the spleen. In humans, mean Hb concentrations of 4.8 g/dl have been reported in infants experiencing acute splenic sequestration (*18*), and this value is similar to the concentrations found in HbS mice (Table 1). Occasionally, RBC trapping leads to vascular occlusion and thrombosis. Many spleen sections contained an organizing thrombus similar to the one shown for HbS3 in Fig. 3A. Larger areas of mineralized tissue and amorphous acellular debris were also observed in older animals. This pathology is indicative of infarcts that occurred earlier in the life of the animals and implies that splenic function may decline over time. In humans, recurrent infarcts can result in autosplenectomy in childhood.

In the livers of HbS mice, there was a generalized congestion of the intrahepatic vasculature and sinusoids with aggregates of sickled RBCs (Fig. 3A). Extramedullary hematopoiesis occurred in the sinusoids as indicated by prominent clusters of erythroid precursors. Kupffer cell erythrophagocytosis with concomitant accumulation of hemosiderin is abundant. There is also periportal and subcapsular focal parenchymal necrosis. All of these pathologies are characteristic of

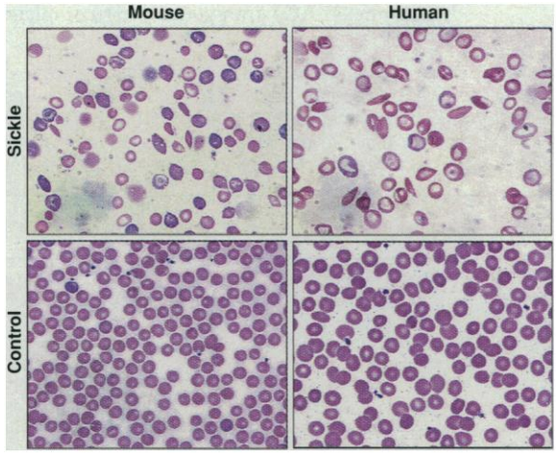

Fig. 2. Microscopic analyses of control and sickle peripheral blood smears from an HbS3 mouse and an individual with sickle cell disease under normal oxygen tension. Original magnification was ×250 in all four panels.

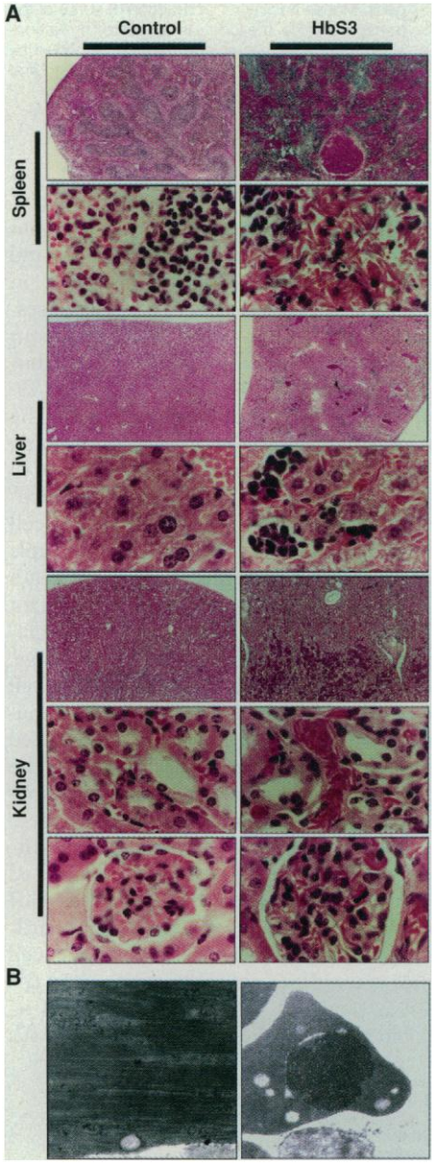

Fig. 3. Histological sections of control and HbS mouse tissues derived from mice under normal oxygen tensions. (**A**) Low magnification (original, ×10) and high magnification (original, ×250) of spleen, liver, and kidney tissue sections (*25*). HbS spleens are characterized by massive expansion of erythroid precursors, pooling of sickled erythrocytes in the sinusoids, vascular occlusion, and thrombosis. Livers of HbS animals exhibit extensive periportal and subcapsular focal necrosis, extramedullary hematopoiesis, and numerous sickled erythrocytes in the intrahepatic vasculature and sinusoids. In the kidneys of HbS mice, vascular occlusion is most prominent in the corticomedullary junction where dilated capillaries are engorged with sickled RBCs. Tubular damage results in a decrease of urine-concentrating ability (Table 1). Histopathology from HbS lines 1 through 6 was similar. (**B**) Electron micrographs of splenic erythroid cells containing human HbS fibers (longitudinal and cross sections, respectively) (*26*). Original magnification was ×20,000.

the liver in human sickle cell patients (*12*).

In the kidneys of HbS mice, engorgement and occlusion of blood vessels with sickled cells causes vascular, tubular, and glomerular changes (Fig. 3A). Vascular occlusion was most prominent in the corticomedullary junction where engorged and dilated capillaries are easily observed. In humans, reduced blood flow through the medullary region frequently causes extensive tubular damage resulting in hyposthenuria or the loss of urine-concentrating ability. When HbS3 mice were deprived of water for only 4 hours, the osmolality of the urine (*21*) was about half of control values (807 ± 285 mOsm compared with 1541 ± 360 mOsm). In younger animals, sickle cell congestion in the glomerulus and increased levels of iron deposition cause a mild membranoproliferative glomerulopathy. There was a moderate multifocal proximal tubular injury resulting in tubular dilatation, epithelial hypoplasia, basement membrane thickening, and iron deposition.

Despite the severe anemia and organ pathology in the HbS mice, more than 90% of the animals survived for 2 to 9 months, and males and females were fertile. The animals described here should be useful for testing drug and genetic therapies designed to treat and ultimately to cure this debilitating disease.

Note added in proof: We have also produced mice that synthesize human HbA, HbF, and recombinant Hbs, exclusively.

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15. LCR $^{A}\gamma$-β^{S} was constructed in a cosmid vector by using deoxyribonuclease I hypersensitive sites 1 through 5 of the human β-globin LCR in a 22-kb Sal I–Cla I fragment [T. M. Ryan *et al.*, *Genes Dev.* **3**, 314 (1989)]. The 5.65-kb $^{A}\gamma$-globin gene fragment included in LCR $^{A}\gamma$-β^{S} contains the $^{A}\gamma$-globin gene from a Hind III site at position −1348 to an Eco RI site at position 4309. The 4.1-kb β^{S}-globin gene fragment included in LCR $^{A}\gamma$-β^{S} contains the β-globin gene from a Hpa I site at position −815 to an Xba I site at position 3285. LCR $^{A}\gamma$-β^{S} fragments were excised from the cosmid vector and injected into C57Bl/6 × SJL fertilized mouse eggs. Transgenic founder animals were bred with C57Bl/6 × SJL mice to establish lines.
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19. Globin chain synthesis was performed by a modification of a protocol communicated to us by R. Popp, Oak Ridge National Laboratories. Freshly drawn blood (150 μl) was collected in five volumes of 2% sodium citrate and 0.5% saline and washed three times with 0.85% saline at room temperature. After they were washed, the cells were resuspended in 1 ml of translation mix and incubated with shaking for 90 min at 37°C, 5% CO_2. The translation mix was 0.5× Dulbecco's modified Eagle's medium F-12 Ham base (leucine-free, Sigma), 0.22% $NaHCO_3$, transferrin (50 μg/ml, Sigma), $Fe(NH_4)_2(SO_4)_2$ (0.25 mg/ml), ^{3}H-leucine (0.5 mCi/ml, Amersham), and the pH was adjusted to 7.1. Cells were washed three times with 0.85% saline after incubation and subsequently lysed in 5 mM phosphate, 0.5 mM EDTA, pH 7.4. Hemolysates were subjected to HPLC as described (*22*). To determine α/non-α ratios, we collected fractions every 20 s, and the radioactivity in each fraction was detected with a Beckman LS 6500 liquid scintillation counter.
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21. Food and water were withheld for 4 hours and urine was collected from 4- to 4.5-month-old mice. Urine osmolality was measured on a Wescor model 5130C vapor pressure osmometer.
22. Reverse-phase HPLC was performed on a Rainin Dynamax system with a Vydac C4 column (25 cm by 0.46 cm). Buffer A was 10% acetonitrile (ACN)/0.1% trifluoroacetic acid (TFA), and buffer B was 90% ACN/0.1% TFA. A nonlinear gradient for 1 hour was used starting at 35% B to 36% B in the first minute, followed by an increase to 42% over the next 48 min, maintained at 42% B for 6 min, and returned to 35% B in the final 5 min. The flow rate was kept at 1 ml/min, and the effluent was monitored at 214 nm. Individual globin chains were quantitated with Dynamax HPLC Method Manager software.
23. Reticulocytes were counted manually after supravital staining with 1% new methylene blue (Sigma) for 10 min at 37°C.
24. RBC counts were measured on a Coulter Counter (Model MHR). Hemoglobin concentrations were determined at 540 nm by the cyanmethemoglobin method with a kit from Sigma (catalog no. 525-A). PVCs were determined by centrifugation of EDTA-treated blood in a Jorvet J503 centrifuge.
25. Tissues were fixed in 70% alcoholic formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin by standard methods.
26. Spleen from a HbS mouse was fixed in 1% buffered glutaraldehyde and then fixed in 1% osmium tetroxide. After dehydration, the sample was embedded in

polybed, sectioned, and stained with uranyl acetate and lead citrate. Ultra-thin sections were examined and photographed with the Hitachi-7000 transmission electron microscope.

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Transgenic Knockout Mice with Exclusively Human Sickle Hemoglobin and Sickle Cell Disease

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To create mice expressing exclusively human sickle hemoglobin (HbS), transgenic mice expressing human α-, γ-, and β^S-globin were generated and bred with knockout mice that had deletions of the murine α- and β-globin genes. These sickle cell mice have the major features (irreversibly sickled red cells, anemia, multiorgan pathology) found in humans with sickle cell disease and, as such, represent a useful in vivo system to accelerate the development of improved therapies for this common genetic disease.

A single base pair change in codon 6 of the β-globin gene causes sickle cell anemia in individuals who are homozygous for the mutation (*1*). Sickle hemoglobin [HbS ($\alpha_2\beta^S{}_2$)] undergoes polymerization upon deoxygenation, thereby distorting erythrocytes into a variety of sickled shapes, damaging the erythrocyte membrane, and ultimately causing anemia, ischemia, infarction, and progressive organ dysfunction. Despite the impressive body of knowledge that has accumulated (*2*), many aspects of sickle cell disease are still poorly understood and treatment options remain limited. Because of the inhibitory effects of mouse α- and β-globin on sickling, transgenic mice expressing various sickle hemoglobins (HbS, HbSAD, HbS-Antilles) develop almost none of the clinical manifestations of sickle cell disease (*3*). Some sickle cell disease pathology has been reported in transgenic mice bred to produce higher concentrations of the "supersickling" hemoglobins (HbSAD and HbS-Antilles) (*4*); however, these animals still lack important features that are commonly found in humans with sickle cell disease (*5*). To overcome these limitations, we have created mice that no longer express mouse α- and β-globin; instead, they express exclusively human α- and β^S-globin.

Three fragments of human DNA were coinjected into fertilized mouse eggs to generate transgenic founders expressing human α- and β^S-globin (*6*). Because γ-globin has antisickling properties, we included the $^G\gamma$- and $^A\gamma$-globin genes to decrease the likelihood that erythrocytes would sickle during gestation and cause fetal death. In the particular transgenic line that was generated [Tg(HuminiLCR$\alpha 1^G\gamma^A\gamma\delta\beta^S$)], $^G\gamma$- and $^A\gamma$-globin are expressed during the embryonic and fetal stages of development and not in adult mice (Fig. 1A) (*7*). Through successive rounds of breeding with knockout mice heterozygous for deletions of the murine α- and β-globin genes, $Hba^0//+$ $Hbb^0//+$ (*8*, *9*), mice homozygous for the α- and β-globin deletions and containing the sickle transgene were generated—Tg(Hu-miniLCR$\alpha 1^G\gamma^A\gamma\delta\beta^S$) $Hba^0//Hba^0$ $Hbb^0//Hbb^0$, hereafter called sickle cell mice (*10*). Many of these mice turned purple and died a few hours after birth; their death was apparently a result of hypoxia brought about by respiratory distress. Because γ-globin concentrations are relatively low [range, 4 to 26% ($\gamma/\gamma+\beta^S$)] in newborn sickle cell mice (Fig. 1B) compared with newborn humans, it is likely that these deaths are caused by the sickling of erythrocytes during the critical period just after birth when the lungs must begin the task of supplying oxygen. Sickle cell mice that survived this early critical period were able to reach adulthood (many are now more than 7 months old) with normal appearance, activity, and fertility (*11*). Erythrocytes in adult sickle cell mice contain exclusively human α- and β^S-globin (Fig. 1B). There is an excess of α-globin chain synthesis (α/β^S, 1.26 ± 0.02;

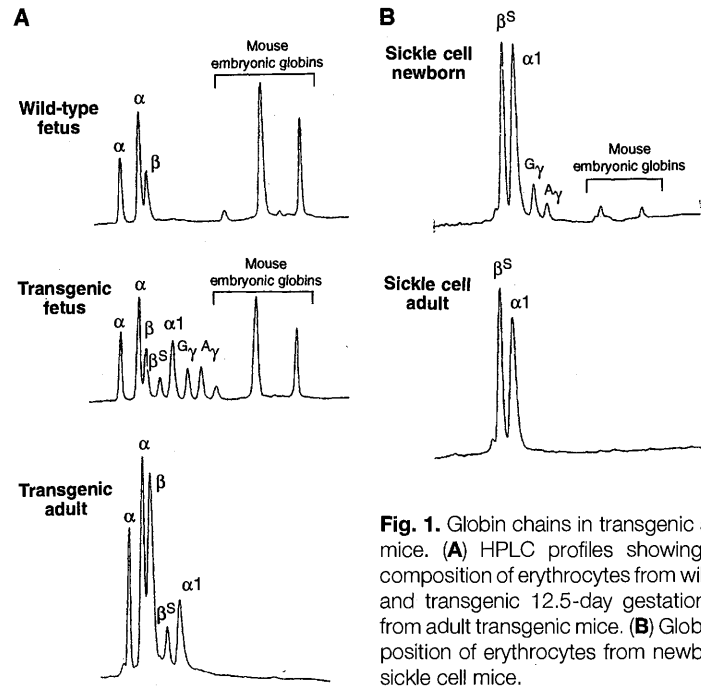

Fig. 1. Globin chains in transgenic and sickle cell mice. (**A**) HPLC profiles showing globin-chain composition of erythrocytes from wild-type (+//+) and transgenic 12.5-day gestation fetuses and from adult transgenic mice. (**B**) Globin-chain composition of erythrocytes from newborn and adult sickle cell mice.

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