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Genetics

High expression of human β^S- and α-globins in transgenic mice: Hemoglobin composition and hematological consequences

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ABSTRACT **A line of transgenic mice ($\alpha^H\beta^S$-11; where α^H is human α-globin) was created in which the human β^S and human α2 globin genes, each linked to the β-globin locus control region, were cointegrated into the mouse genome. On a normal genetic background, the transgenic mice produced 36% human β^S-globin chains with an α^H/β^S ratio of 1.3. Higher levels of β^S were achieved by breeding the transgenic mice with mutant mice carrying a mouse β^{major}-globin gene deletion. Mice heterozygous for the β^{major} deletion ($\alpha^H\beta^S[\beta^{MD}]$; MD, mouse deletion) had 54% β^S with an α^H/β^S ratio of 1.0; mice homozygous for the β^{major} deletion ($\alpha^H\beta^S[\beta^{MDD}]$) had 72.5% β^S and an α^H/β^S ratio of 0.73. Because mouse α chains inhibit hemoglobin (Hb) S polymerization, we bred the mice to heterozygosity for a mouse α-globin deletion. These mice ($\alpha^H\beta^S[\alpha^{MD}\beta^{MDD}]$) had an increased α^H/β^S ratio of 0.89 but expressed 65% β^S. Expression of the human genes cured the thalassemic phenotype associated with the murine β^{major} deletion. Transgenic $\alpha^H\beta^S[\beta^{MDD}]$ mice had normal hematocrit and Hb and somewhat elevated reticulocytes (6% vs. 3% for control), whereas the mice carrying the α-globin deletion ($\alpha^H\beta^S[\alpha^{MD}\beta^{MDD}]$) had a normal hematocrit and Hb and more elevated reticulocytes (10.3 ± 7.6% vs. 3.4 ± 1.0%). Expression of the transgene restored a normal distribution of erythrocyte densities when compared to thalassemic mice; however, the average mean corpuscular Hb concentration of $\alpha^H\beta^S[\beta^{MDD}]$ mice increased to 35.7 g/dl (vs. control 33.7 g/dl) whereas that of $\alpha^H\beta^S[\alpha^{MD}\beta^{MDD}]$ mice was further elevated to 36.3 g/dl. The intrinsic oxygen affinity was increased in transgenic mouse erythrocytes at 280 milliosmolal, and the Po_2 at midsaturation of $\alpha^H\beta^S[\alpha^{MD}\beta^{MDD}]$ erythrocytes was higher than that of $\alpha^H\beta^S[\beta^{MDD}]$ cells (37.4 ± 2 vs. 33.5 ± 1 mmHg). The higher values of the mean corpuscular Hb concentration and intrinsic Po_2 at midsaturation, which favor *in vivo* sickling, may explain the slightly more severe hematological picture in $\alpha^H\beta^S[\alpha^{MD}\beta^{MDD}]$ mice. We conclude that the transgenic mouse with high Hb S expression does not exhibit adult anemia but does have abnormal hematological features: increased erythrocyte density, high oxygen affinity, and reticulocytosis with increased stress reticulocytes.**

An animal model of sickle cell disease, which would allow testing of experimental hypotheses and interventions that are not possible in humans, has been a long-standing goal. Progress has been reported by several groups. Greaves *et al.* (1) introduced a construct that contained a locus control region (LCR) in tandem with two human α1 genes and a single human β^S gene, generating one transgenic mouse that expressed high levels of human β^S and human α chains, most of whose erythrocytes (RBCs) were able to sickle *in vitro* but that was not anemic; however, the founder was not propagated. Ryan *et al.* (2) cointroduced two constructs consisting of the β^S gene or the human α1 gene, each in tandem with a LCR, generating a transgenic line expressing 51% human hemoglobin (Hb) S, on a heterozygous β-thalassemic background, in which most cells sickled *in vitro*. Rubin *et al.* (3) have described a transgenic line that expressed the low oxygen affinity mutant Hb $S^{Antilles}$. In contrast to sickle trait patients, patients with Hb $S^{Antilles}$ trait exhibit symptoms of vasoocclusion due to a combination of the low solubility and low oxygen affinity of this mutant Hb. The animals studied, which were heterozygous for the mouse β^{major} deletion, expressed ≈50% of their β-globin as $\beta^{S\text{-}Antilles}$; however, the ratio of human α-globin (α^H)/$\beta^{S\text{-}Antilles}$ was $\ll$1. These mice were slightly anemic and exposure to hypoxia resulted in increased numbers of irreversibly sickled cells (ISCs). Trudel *et al.* (4) reported the development of a mouse strain bearing α^H and β^{SAD} ($\beta^{S\text{-}Antilles\text{-}D\ Punjab}$) genes, which on a heterozygous β-thalassemic background express 26% Hb SAD. These mice displayed increased fetal and neonatal wastage, anemia during neonatal development (but not as adults), reticulocytosis, enlarged spleen, and sensitivity to hypoxia, as well as ISCs *in vivo* and sickling *in vitro*.

We report here production and characterization of transgenic mice carrying human LCR–β^S and human LCR–α2 constructs cointegrated in the genome. The original transgenic mice were bred to mice with deletions of mouse β^{major} or α genes to increase expression of human globins and reduce mouse globins. These mice were either homozygous for β^{major} deletion (β^{MDD}; MD, mouse deletion) or also heterozygous for an α-globin deletion (α^{MD}) and had a normal mean corpuscular Hb (MCH), no anemia, reticulocytosis, a small number of ISCs, and an increased mean corpuscular Hb concentration (MCHC).

In ref. 5, we report observations on organ damage under ambient conditions and the induction of hematological and renal abnormalities by hypoxia. A preliminary report of this work has appeared (6).

METHODS

Production of Transgenic Mice. The LCR–α2 gene (4) was constructed by excising the 3.5-kilobase *Sac* I–*Sac* I fragment including the human α2 gene from plasmid pVhα2 [a gift of R. Gelinas (Icos Corporation, Bothell, WA); originally derived from λHαG1 (8)]. The DNA was treated with the Klenow fragment of DNA polymerase to blunt the ends and ligated to the plasmid pBluescript KS linearized with *Sma* I. A clone containing the α2 gene in the same orientation as the

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Abbreviations: LCR, locus control region; Hb, hemoglobin; Hct, hematocrit; MCHC, mean corpuscular Hb concentration; RBC, erythrocyte; ISC, irreversibly sickled cell; MCH, mean corpuscular Hb; mosm, milliosmolar; IEF, isoelectric focusing; HS, DNase I-hypersensitive; p50, Po_2 at midsaturation; C_{sat}, concentration of deoxyHb in equilibrium with the polymer; SS, sickle cell anemia; SC, double heterozygote with both Hb S and Hb C.

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lacZ gene was identified and was linearized with *Eco*RI and *Sal* I, both of which cut in the polylinker upstream from the $\alpha 2$ gene. The 8-kilobase mini-LCR fragment containing the four 5′ hypersensitive (HS) sites (5) was isolated as an *Eco*RI–*Sal* I fragment and ligated to the linearized α-globin plasmid (Fig. 1). The LCR–β^S construct (7) was digested with *Not* I and *Xho* I and the LCR–$\alpha 2$ construct was digested with *Not* I and *Sal* I to remove plasmid sequences. Each transgene fragment was purified (9) and a mixture of the two fragments at a concentration of 2 μg/ml was injected into fertilized eggs of strain FVB/N.

Mouse Strains. FVB/N inbred mice were obtained from Carl Hansen (National Institutes of Health, Bethesda, MD). Mice carrying the mutant β-globin haplotype $Hbb^{d3(th)}$ (10) were obtained from R. Popp (Oak Ridge National Laboratory) and were backcrossed for a total of eight generations with C57BL/6J mice before being bred to homozygosity for $Hbb^{d3(th)}$. C57BL/6J mice congenic for the α-globin mutation $Hba^{th\text{-}J}$ were obtained from The Jackson Laboratory.

Hematological Measurements. Both manual procedures [hematocrit (Hct), Hb, MCHC, and ISC count] and a Technicon H1 (MCH, mean corpuscular Hb, and MCHC) were used. Stress reticulocytes had a nucleus-like aggregation of supravital-stained particles, abundant diffuse dots, or a "bicycle-wheel" arrangement. Mature reticulocytes had less than seven particles per cell. ISCs were counted in reticulocyte smears and scanning electron microscopy photographs by using the absence of dimple and a length-to-width ratio of 2 or more.

Oxygen Affinity Determinations [Po_2 at Midsaturation (p50)]. RBCs were washed with 10 mM Hepes (pH 7.4) at 37°C, 5 mM KCl, 5 mM glucose, and NaCl to give 280 milliosmolar (mosm). O_2 equilibrium curves were obtained at 37°C on a SLM Aminco O_2 dissociation analyzer at a Hct of 10–20%.

Solubility of Hemolysates of $\alpha^H\beta^S[\beta^{MDD}]$ and $\alpha^H\beta^S[\alpha^{MD}\text{-}\beta^{MDD}]$ RBCs Under Deoxygenated Conditions. Concentration of deoxyHb in equilibrium with the polymer (C_{sat}) was determined in a 0.1 M potassium phosphate (pH 7.35) at 25°C. The Hb was first deoxygenated and then sodium dithionite was added to equal three times the final concentration of Hb. Samples were allowed to gel overnight at 25°C and were centrifuged the next day at 25°C for 2 h at 35,000 rpm in a Beckman SW 65 rotor. Purified Hb S in the same buffer was always included as a control. The supernatants were removed anaerobically and Hb concentrations and deoxygenated pH values were determined.

Percoll/Larex Density Gradients. Percoll (colloidal silica coated with polyvinylpyrrolidone; Pharmacia) and Larex (arabinogalactan polysaccharide; Consulting Associates, Tacoma, WA) gradients were formed as described (11).

Chromatographic Separation of Globin Chains. The globin composition was determined by HPLC using a denaturing solvent that separates the globin chains and a Vydac large-pore (300 Å) C_4 column, 4.6 × 250 mm (Separations Group, Hesperia, CA) with an acetonitrile/H_2O/trifluoroacetic acid gradient (12) with 2% less acetonitrile in the starting gradient than was used by Schroeder *et al.* (12) for separating human chains. Mouse α and β chains were identified by digesting the HPLC-separated globin chains with trypsin and identifying the fragments by HPLC.

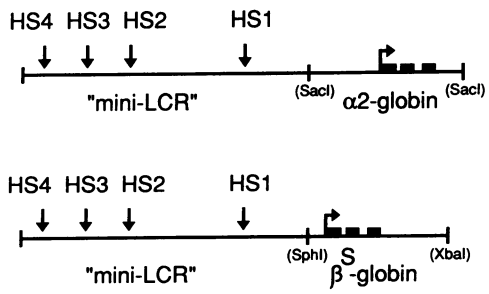

FIG. 1. DNA constructs used to create the transgenic mouse line. Restriction enzyme sites in parentheses indicate the ends of the $\alpha 2$- and β^S-globin fragments used. HS1–HS4, DNase I HS sites from the human β-globin 5′ LCR.

Isoelectric Focusing (IEF). Hbs were separated by IEF with the Isolab Resolve-Hb kit. The percent of each band was determined by scanning at 560 nm using a Beckman gel scanner. For HPLC, bands were cut out and eluted with distilled water.

RBC Morphology. Scanning electron microscopy of RBCs was performed as described (13). The RBC distribution width was determined by a Coulter S+IV counter.

Statistics. Statistical calculations were made using the program STATGRAPHICS (Statistical Graphics Corporation, Rockville, MD) Version 5.0 and are expressed as mean ± SD and number of individuals studied (n).

RESULTS

Generation of Transgenic Mice Coexpressing Human β^S and Human α-Globin Chains. The constructs in Fig. 1 were employed to obtain transgenic mice. Each contained the major 5′ HS sites of the human β-globin LCR (14–16) joined to form a "mini-LCR" (7) and either human $\alpha 2$ or human β^S gene. LCR constructs with the four 5′ HS sites have been shown to greatly enhance the expression of linked α- or β-globin genes in transgenic mice (17–20). The LCR–$\alpha 2$ and LCR–β^S constructs coinjected into mouse zygotes and three of the 20 mice born were shown by Southern blot analysis (data not shown) to carry multiple copies of both genes.

Each of the three transgenic founders was mated with wild-type mice of the same inbred strain (FVB/N), and the F_1 progeny were screened for inheritance of the transgenes by DNA and/or Hb analysis. Only one of the three founder mice, mouse 11, transmitted the transgenes to a fraction of its progeny, and the transgenic line described in this report was derived from this animal. The LCR–$\alpha 2$ and LCR–β^S transgenes were coinherited in all subsequent generations of this transgenic line, indicating that they had cointegrated at the same locus.

On mice of the inbred FVB/N genetic background (homozygous for the wild-type Hbb^d haplotype), the level of human β^S globin chains in RBCs was 36% of all β chains, and the ratio of human α- to β-globin chains was 1.35. When heterozygous transgenic mice were crossed to each other, no apparent homozygotes with higher levels of β^S were observed among the progeny.

Increased Level of Human β^S-Globin in Transgenic Mice with Mouse β^{major} Deletion. To increase human β^S-globin chains vs. endogenous mouse β-globin chains, the transgenic mice were bred with mice carrying the $Hbb^{d3(th)}$ mutation (10) on the C57BL/6J background. This mutant haplotype contains a deletion of the *Hbb-b1* gene (which normally encodes β^{major}-globin), but an intact *Hbb-b2* gene, encoding β^{minor}-globin. Homozygotes synthesize only β^{minor} chains, and although they increase synthesis of β^{minor} chains, they are deficient in the overall level of β-globin synthesis (8). In transgenic mice heterozygous for this mutation ($\alpha^H\beta^S[\beta^{MD}]$), 54% of all β chains were human β^S, and the ratio of human α- to β^S-globin decreased to 1 (i.e., equal numbers of human α and β chains were synthesized). On a homozygous $Hbb^{d3(th)}$ background ($\alpha^H\beta^S[\beta^{MDD}]$), the expression of β^S increased again to 72.5 ± 2.4% of all β chains; however, the ratio human α to β chains decreased to 0.73 ± 0.06.

Increased Level of Human α-Globin in Transgenic Mice Heterozygous for a Deletion of the Mouse α-Globin Locus. Hoping to further decrease the level of endogenous mouse

Table 1. Chain and Hb composition in mouse RBCs

Mouse	Chain composition		Symmetric tetramer, %			
	% β^S	% α^H	$(\alpha^M\beta^S)_2$	$(\alpha^M\beta^M)_2$	$(\alpha^H\beta^S)_2$	$(\alpha^H\beta^M)_2$
$\alpha^H\beta^S[\beta^{MDD}]$	72.7 ± 2.4 (64)	44.7 ± 2.4 (64)	29.4 ± 1.4	14.9 ± 2.1	41.9 ± 2.7	11.9 ± 1.8
$\alpha^H\beta^S[\alpha^{MD}\beta^{MDD}]$	65.1 ± 8.5 (24)	53.1 ± 6.2 (24)	18.4 ± 1.4	16.8 ± 2.1	40.6 ± 2.6	20.4 ± 1.4

Chains were separated by HPLC; data in parentheses are number of animals. Percent symmetric tetramers are the average ± SD of six determinations by IEF under oxygenated conditions.

Hb and increase the amount of human Hb S formed, we next bred these transgenic mice with mice heterozygous for a deletion of the entire α-globin locus *Hba*$^{th\text{-}J}$. Unlike the mutant β-globin haplotype *Hbb*$^{d3(th)}$, the *Hba*$^{th\text{-}J}$ haplotype (and all existing murine α-thalassemia mutations) cannot be bred to homozygosity, presumably because the deletion includes other essential genes (21). However, the heterozygous state should halve the dosage of mouse α-globin genes and hence the synthesis of endogenous α-globin chains. In mice that were homozygous for *Hbb*$^{d3(th)}$ and heterozygous for *Hba*$^{th\text{-}J}$ ($\alpha^H\beta^S[\alpha^{MD}\beta^{MDD}]$), the ratio of human α-globin/human β^S was increased to 0.89 ± 0.17, the percent of Hbs containing mouse α chains was reduced, and the percent of human β^S of all β chains decreased to 65.1 ± 6.2%.

Globin Chain Composition. The HPLC results (Table 1 and Figs. 2 and 3) allowed the ratio of α to β chains to be calculated. In the $\alpha^H\beta^S[\beta^{MDD}]$ mouse the ratio of all α chains to all β chains was 1.37 ± 0.15 (n = 20), whereas in the $\alpha^H\beta^S[\alpha^{MD}\beta^{MDD}]$ mouse the ratio of all α chains to all β chains was 1.17 ± 0.19 (n = 9), which reflects the expected decrease in α-chain production due to the α-globin deletion. This difference was statistically significant with $P < 0.005$.

Hb Composition. In both the $\alpha^H\beta^S[\beta^{MDD}]$ and the $\alpha^H\beta^S[\alpha^{MD}\beta^{MDD}]$ mice, four bands can be identified by IEF. These represent the four possible symmetrical tetramers formed from the dimers $\alpha^M\beta^S$, $\alpha^M\beta^M$, $\alpha^H\beta^S$, and $\alpha^H\beta^M$, which are present in the values in Table 1.

Oxygen Affinity of Transgenic Mouse RBCs. Mouse RBCs expressing high levels of α^H and β^S had a higher oxygen affinity than normal mouse RBCs. The p50 value and 2,3-bisphosphoglycerate content for various constructs and control mice are given in Table 2 and plotted in Fig. 4. At the highest percent β^S, the mouse p50 averages 33.5 mmHg (1 mmHg = 133 Pa) and approaches the p50 of normal human Hb, 25 mmHg. Since these measurements were made at 280 mosm, which is hypotonic for mouse RBCs, it is unlikely that polymerization occurred during the measurement. Hence, the p50 values correspond to the intrinsic oxygen affinity of these RBCs independent from polymerization.

C_{sat} of Transgenic Mouse Hbs. The Hb concentration in equilibrium with the polymer at room temperature was measured for ($\alpha^H\beta^S[\alpha^{MD}\beta^{MDD}]$) and ($\alpha^H\beta^S[\alpha^{MD}\beta^{MDD}]$) mice and values of 29.6 ± 1.5 g/dl and 30.9 ± 1.5 g/dl, respectively, were obtained. These values are not statistically different at the current number of samples.

RBC Densities. Density gradients demonstrated that expression of human α and β^S restored a normal density distribution in $\alpha^H\beta^S[\beta^{MDD}]$ mice. RBC density was compared for control mice and transgenic mice under physiological conditions. The MCHC in $\alpha^H\beta^S[\beta^{MDD}]$ mice increased to 35.1 g/dl over the 33.7 g/dl of the control mice (C57BL/6J) whereas that of the $\alpha^H\beta^S[\alpha^{MD}\beta^{MDD}]$ mice increased to 36.3 g/dl (Fig. 5 and Table 2). The density gradients of homozygous β-thalassemic (Hbb$^{d3(th)}$) nontransgenic mice exhibit a characteristically broad density distribution that is different from that of the transgenic mice. The RBC distribution width, a measure of RBC heterogeneity, was higher for the transgenic than control animals (Table 2).

Hct, Reticulocytes, and ISCs. More than 100 transgenic mice expressing various levels of β^S have been examined (Table 2). The $\alpha^H\beta^S[\beta^{MDD}]$ mice were found to have an average Hct of 47.5 ± 3% (n = 83), which was similar to that found for C57BL/6J mice, 46.5 ± 2.1% (n = 21). Furthermore, the MCH was normal, and the Hb was slightly elevated. These results clearly indicate that the thalassemic phenotype seen in homozygous β-thalassemic mice with no transgene (Hct, 33.7 ± 2.83%; n = 14) is completely cured. The reticulocyte count of $\alpha^H\beta^S[\beta^{MDD}]$ mice, 6.7 ± 2% (n = 23), was elevated compared to that for control C57BL/6J mice of 3.3 ± 1% (n = 5) and somewhat higher than that of FVB/N mice (the original founder strain), which had reticulocyte counts of 5.2 ± 2.7% (n = 6). The $\alpha^H\beta^S[\alpha^{MD}\beta^{MDD}]$

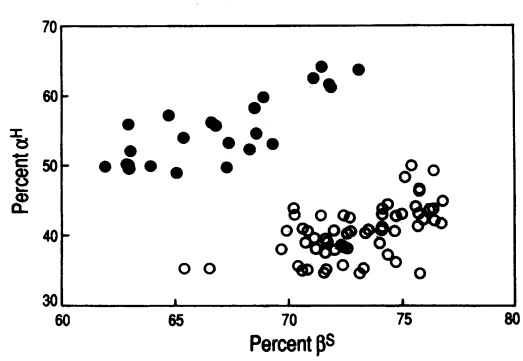

FIG. 2. Percent α^H vs. percent β^S as determined by HPLC for $\alpha^H\beta^S[\beta^{MDD}]$ (open circles) and $\alpha^H\beta^S[\alpha^{MD}\beta^{MDD}]$ mice (solid circles). Note that, although there is considerable variation among mice with the same background, mice with different backgrounds do not overlap.

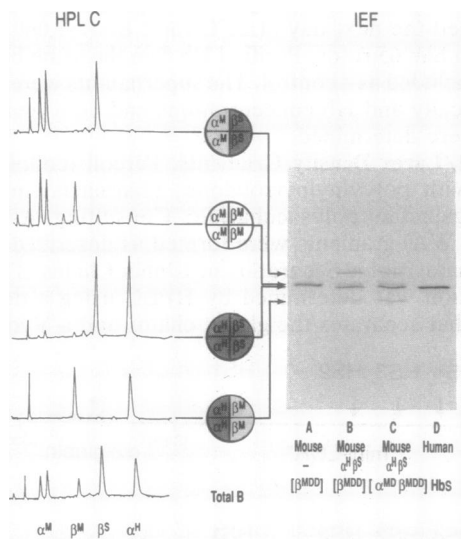

FIG. 3. HPLC and IEF analyses of Hb from $\alpha^H\beta^S[\beta^{MDD}]$ and $\alpha^H\beta^S[\alpha^{MD}\beta^{MDD}]$ mice. IEF evaluates the dimers and HPLC evaluates the globins both in the total hemolysate (Total B) and in the individual IEF bands. The shaded portions of the circles indicate the human component of the four tetramers present in IEF. Note that the number of Hb species present is the same for both mouse backgrounds but that the percentage of the heterodimers differs between the two backgrounds.

Table 2. Hematological attributes of mice expressing β^S- and α^H-globin

Parameter	C57BL/6J	$\alpha^H\beta^S[\beta^{MDD}]$	$\alpha^H\beta^S[\alpha^{MD}\beta^{MDD}]$
Hct, %	46.5 ± 2.1 (21)	47.0 ± 2.4 (78)	45.9 ± 1.2 (23)
Hb, g/dl	15.9 ± 0.8 (2)	17.4 ± 1.3 (4)	17.3 ± 1.4 (4)
Reticulocytes, %	3.4 ± 1.0 (4)	6.7 ± 1.7 (22)	10.3 ± 7.6 (6)
ISCs, %	—	2.4 ± 0.5 (5)	1.4 ± 1.5 (3)
RDW, %	13.1 ± 0.2 (4)	16.0 ± 1.8 (6)	14.7 ± 0.3 (4)
MCHC, g/dl	33.7 ± 0.4 (2)	35.1 ± 1.7 (4)	36.3 ± 1.4 (3)
MCH, pg per cell	14.5 ± 1.0 (5)	14.1 ± 0.5 (7)	14.3 ± 0.2 (7)
MCV, fl	45.4 ± 0.9 (5)	43.0 ± 1.4 (7)	41.9 ± 0.8 (7)
p50, mmHg	41.5 ± 2 (4)	33.5 ± 1 (4)	37.4 ± 2 (4)
DPG, μmol/g of Hb	28.6 ± 1 (2)	26.7 ± 2 (2)	26.9 ± 2 (4)

Data are the mean ± SD. Values in parentheses are the number of animals tested. RDW, RBC distribution width; MCV, mean corpuscular volume; DPG, 2,3-bisphosphoglycerate.

mice had more elevated reticulocyte counts (10.3 ± 7.6%, n = 6) than control mice or other β^S mice. All transgenic mice had a slightly elevated Hb (17.3 ± 1.4 g/dl vs. 15.9 ± 0.8 g/dl for control mice), possibly to compensate for the higher oxygen affinity of cells containing human Hb, which may result in lower tissue oxygenation. They also exhibited an increased number of the stress reticulocytes: $\alpha^H\beta^S[\beta^{MDD}]$ (2.5 ± 0.6%, n = 18), $\alpha^H\beta^S[\alpha^{MD}\beta^{MDD}]$ (2.9 ± 1.6%, n = 6) vs. C57BL/6J (0.8 ± 0.4%, n = 6) with $P < 2 \times 10^{-6}$ and 8×10^{-3}, respectively.

The transgenic mice had a small number (2–3%) of ISCs. The ISCs were bipointed and had no central concavity but were not severely dehydrated and were reminiscent of the early human ISCs. A plasma of 327 mosm was reported for mice (22) and was confirmed for control and transgenic animals by measuring 20 plasma samples (332 ± 16 mosm).

DISCUSSION

A strain of transgenic mice has been created that carries human β^S- and α-globin gene constructs and expresses high levels of human β^S and α chains in its RBCs. The α^H and β^S transgenes, which were coinjected and closely linked in the mouse genome, were inherited together and stably expressed over several generations. When the transgenes were bred into a genetic background homozygous for a deletion of the endogenous β^{major} gene ($Hbb^{d3(th)}$), the level of human β^S chains was increased ≈2-fold in the resulting $\alpha^H\beta^S[\beta^{MDD}]$ mice. Although nontransgenic mice homozygous for this deletion have the characteristics of β-thalassemia (10), the human β^S transgene clearly "cured" the mouse thalassemia, since $\alpha^H\beta^S[\beta^{MDD}]$ mice had normal MCH, slightly elevated Hb levels, close to normal density distributions (Fig. 5), and nearly normal Hcts. The relative amount of human α-globin chains was increased by introducing a deletion of the mouse α-globin locus (Hba^{th-J}), in the heterozygous state ($\alpha^H\beta^S[\alpha^{MD}\beta^{MDD}]$); these mice also had normal MCH and slightly elevated Hb levels. The presence of the transgene also restored the cell density pattern to normal in contrast to that of the nontransgenic deletional parents.

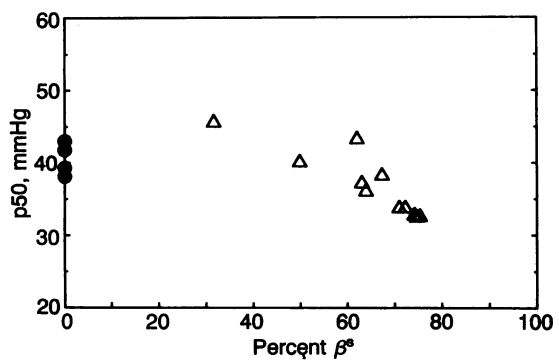

FIG. 4. p50 values of RBCs from control and transgenic mice with different levels of β^S expression plotted as a percent β^S. △, p50 of RBCs from the founder strain, $\alpha^H\beta^S[\beta^{MD}]$, $\alpha^H\beta^S[\beta^{MDD}]$, and $\alpha^H\beta^S[\alpha^{MD}\beta^{MDD}]$; ●, p50 from C57BL/6J control mice.

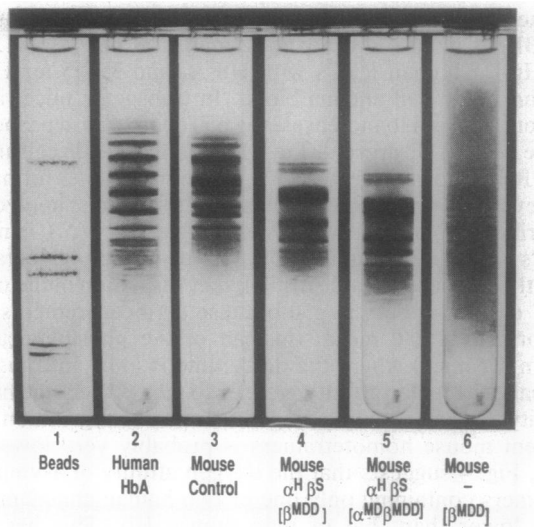

FIG. 5. Percoll/Larex density gradients at 38°C, pH 7.4, and 280 mosm for normal human and mouse (C57BL/6J) RBCs and for $\alpha^H\beta^S[\beta^{MDD}]$, $\alpha^H\beta^S[\alpha^{MD}\beta^{MDD}]$, and $(-)[\beta^{MDD}]$ mice. Note that both transgenic animals have denser RBCs (a higher MCHC) than C57BL/6J mice and that the $\alpha^H\beta^S[\alpha^{MD}\beta^{MDD}]$ mouse has a higher average density than that of the $\alpha^H\beta^S[\beta^{MDD}]$ mouse. Note also that the presence of the human Hb has normalized the width of the density distributions of the two transgenic mice when compared to that of the β-thalassemic mouse $\{(-)[\beta^{MDD}]\}$.

Although mice carrying the transgenes on the various genetic backgrounds were frequently intercrossed, no progeny homozygous for the transgene were detected at any time, suggesting that the transgenic homozygotes are not viable *in utero*. One explanation is that the insertion of the transgene into the mouse genome may have resulted in a recessive lethal mutation, as occurs in ≈10% of transgenic lines (23, 24). However, it is possible that elevated expression of human β^S-globin in homozygous embryos leads to excess polymer formation in the hypoxic environment of the uterus. The observation that no homozygous transgenic mice were obtained for several lines expressing Hb SAD (ref. 4 and F.C., unpublished data) tends to favor the latter explanation.

The percent α^H and β^S detected by HPLC varied significantly among mice of the same background, which may account for differences in the hematological and physiological severity observed in these animals. IEF indicated the presence of four symmetrical tetramers that correspond to Hb S $(\alpha^H\beta^S)_2$, mouse Hb $(\alpha^M\beta^M)_2$, and tetramers of the heterodimers ($\alpha^H\beta^M$ and $\alpha^M\beta^S$). We use the term heterodimer to denote dimers formed from globin chains of different species. Since these four types of dimers can freely combine, six additional asymmetric tetramers are formed in solution. As predicted by Bookchin and Nagel (25) based on Hb C^{Harlem} studies and further supported by the results of Sunshine *et al.* (26), tetramers with only one β^S chain can be incorporated into the polymer, although with a lower probability. In the transgenic mouse, incorporation of asymmetric tetramers will be more complicated because some tetramers also contain mouse α chains.

The p50 calculated from the oxygen equilibrium curve of C57BL/6J mouse RBCs was 42 mmHg at pH 7.2 vs. 26–28 mmHg for human RBCs with Hb A, and 35–55 mmHg for human sickle cell anemia blood. In transgenic mice, as the percent human Hb increases, the p50 decreases in proportion to the percent β^S present (Fig. 4). In human sickle cell anemia (SS) RBCs, low oxygen affinity is due to polymer formation; however, in mouse RBCs elevated 2,3-bisphosphoglycerate contributes significantly to low oxygen affinity (27); in mouse RBCs containing human Hb, 2,3-bisphosphoglycerate is slightly decreased (Table 2). Polymer formation in the mouse RBC did not affect the p50 because all measurements were performed at 280 mosm (instead of the physiological 330 mosm for mice) where the delay time is long. In transgenic animals, the lower-affinity mouse Hb may protect the higher-affinity tetramers containing β^S from deoxygenation. The percent mouse homotetramers is probably very low; however, Fig. 4 suggests that the oxygen affinity of asymmetric tetramers containing only one or two human chains may be even lower than that of pure mouse Hb. This has been demonstrated for the hybrid tetramer containing human α and mouse β^{Single} chains (4).

The deoxyHb concentration in equilibrium with the polymer at room temperature (C_{sat}) was measured for transgenic mice ($\alpha^H\beta^S[\beta^{MDD}]$ or $\alpha^H\beta^S[\alpha^{MD}\beta^{MDD}]$) and values of 29.6 ± 1.5 g/dl and 30.9 g/dl (at 25°C) were obtained, respectively. These values should be compared with the C_{sat} of a Hb A and Hb S mixture similar to that of sickle trait patients under the same conditions (27 g/dl) and contrasted with the C_{sat} of homozygous SS RBCs of 17 g/dl under the same conditions. The C_{sat} for a 50:50 mixture of Hb S and Hb F was similar to that of a 50:50 mixture of Hb S and C57BL/6J mouse Hb, which suggests that mouse Hb may be as inhibitory to polymerization as Hb F. It cannot be predicted *a priori* whether the tetramer composition of $\alpha^H\beta^S[\beta^{MDD}]$ or $\alpha^H\beta^S[\alpha^{MD}\beta^{MDD}]$ mice will be more favorable to polymer formation.

The polymer solubility (C_{sat}) described here is for a hemolysate and will not reflect the effect of the high and variable MCHC of mouse RBCs observed *in vivo* nor the effects of pH and osmolarity. The elevated MCHC observed for all transgenic animals and the further increase consistently observed for $\alpha^H\beta^S[\alpha^{MD}\beta^{MDD}]$ mice suggests that cation transport is perturbed in mice expressing high levels of β^S. The elevated MCHC will promote more sickling in the $\alpha^H\beta^S[\alpha^{MD}\beta^{MDD}]$ animals. The complexities of predicting the probability of vasoocclusion are highlighted by the observed organ damage in this transgenic mouse line (see ref. 5).

In conclusion, transgenic $\alpha^H\beta^S$ mice that were homozygous for a mouse β^{major}-globin deletion ($\alpha^H\beta^S[\beta^{MDD}]$) had normal Hb and somewhat elevated reticulocyte counts (6 vs. 3% for control mice), whereas mice that were concomitantly heterozygous for an α-globin deletion ($\alpha^H\beta^S[\alpha^{MD}\beta^{MDD}]$) had a normal Hb and more elevated reticulocyte counts (10%). The number of stress or young reticulocytes was doubled, which is generally associated with erythropoietic stress. The number of reticulocytes may be particularly relevant in these transgenic animals since younger RBCs are more likely to adhere (24, 28, 29), and adhesion to postcapillary venules may provide a long enough exposure to a low oxygen environment to allow the long polymerization delay time to be overcome. A small number of ISCs were seen in both strains of transgenic mice, which supports the contention that *in vivo* polymerization occurs. The elevated MCHC seen in $\alpha^H\beta^S[\beta^{MDD}]$ mice is probably not due to excess α chains since the effect is even larger in $\alpha^H\beta^S[\alpha^{MD}\beta^{MDD}]$ mice, which have a smaller excess of α chains.

Although C_{sat} values suggest that pathology in these mice should be similar to that for sickle trait individuals, the value reflects an average Hb composition. *In vivo*, the diversity of RBC Hb concentration (MCHC) and increased percent of young and mature reticulocytes, the high normal plasma osmolarity, which is characteristic of mice, and the response to low pH and even higher osmolarity (as for example, in the kidney) may create conditions that would allow sickling to proceed at a rate compatible with vasoocclusion. Hence, predictions of the probability of vasoocclusion *in vivo* from simple C_{sat} data without regard to physiological conditions are misleading.

The hematological changes reported here complement the observation of pathological changes in organs of the transgenic animals (see ref. 5) providing evidence that *in vivo* vasoocclusion does occur in this transgenic line of mice expressing the β^S gene.

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