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Research Article

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Hypoxia-induced In Vivo Sickling of Transgenic Mouse Red Cells

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Abstract

To develop an animal model for sickle cell anemia, we have created transgenic mice that express a severe naturally occurring human sickling hemoglobin, Hb S Antilles. Due to its low solubility and oxygen affinity, Hb S Antilles has a greater propensity to cause red cell sickling than Hb S. To make transgenic animals that express a high level of Hb S Antilles, the erythroid-specific DNase I hypersensitive site II from the human β -globin cluster was linked independently to the human α 2-globin gene and to the β^S Antilles gene. Embryos were injected with both constructs simultaneously and seven transgenic mice were obtained, three of which contained both the human α and the human β^S Antilles transgene. After crossing the human transgenes into the mouse β -thalassemic background a transgenic mouse line was derived in which approximately half the β -globin chains in the murine red cells were human β^S Antilles. Deoxygenation of the transgenic red cells in vitro resulted in extensive sickling. An increase of in vivo sickling was achieved by placing these transgenic mice in a low oxygen environment. This murine model for red cell sickling should help to advance our understanding of sickle cell disease and may provide a model to test therapeutic interventions. (*J. Clin. Invest.* 1991. 87:639–647.) Key words: hemoglobin S Antilles • animal model • sickle cell anemia • hemoglobin polymerization

Introduction

A transgenic mouse model for sickle cell anemia would be valuable in the analysis of factors that initiate red cell sickling in the whole organism, in the study of the pathophysiological consequences of sickling, and in the development of experimental approaches for treatment. Prior attempts at creating such a model have included the introduction of the human β^S -(1) and both the human α - and β^S -globin genes (2, 3) into the mouse genome. The transgenic mouse with the highest levels of Hb S in its red cells (> 80% of red cell hemoglobin was Hb S) was described by Greaves et al. (2). Although complete deoxygenation resulted in 100% of red cells from this animal to sickle in vitro, only 0.1% of red cells sickled in vivo. Analysis of the animal revealed normal somatic development, no obvious manifestations of disease, and no evidence of hemolytic ane-

mia despite the amount of Hb S. The failure of this mouse to exhibit signs of significant in vivo sickling, taken in conjunction with prior analyses of the mechanism regulating hemoglobin polymerization, suggests that criteria for significant in vivo sickling to occur may need to include the following: (a) that the pathological sickling hemoglobin molecules be present at sufficiently high levels or have sufficiently low solubility such that polymerization and sickling will occur in the presence of murine hemoglobin (1, 4), and (b) that the human hemoglobin have an oxygen affinity that will favor its existence in the deoxygenated form in murine red cells.

Hemoglobin S Antilles (Hb S Antilles)¹ is a human sickling variant that, unlike Hb S, results in significant red cell sickling, anemia, and vaso occlusion in heterozygote carriers (A/S Antilles) (5). Erythrocytes of carriers of the Hb A/S Antilles trait sickle at an oxygen partial pressure similar to that which induces sickling in Hb S/C disease. The β^S Antilles-globin chain of the Hb S Antilles molecule contains two mutations, Glu₆ → Val₆ (the β -chain mutation responsible for sickle cell anemia) and Val₂₃ → Ile₂₃ (5). These two mutations result in the decreased solubility and lower oxygen affinity of Hb S Antilles compared with Hb S. (For purified Hb S Antilles and Hb S the minimal gelling concentration is 11 g/dl and 18.4 g/dl, and the P₅₀ is 9 mmHg and 5.5 mmHg, respectively.) Red cells of transgenic mice expressing high levels of human globin transgenes also contain highly soluble murine hemoglobin (7–9). The oxygen affinity of the murine hemoglobin is significantly lower than that of Hb S but not that of Hb S Antilles (6). These observations suggest that Hb S Antilles may be a better candidate for producing in vivo red cell sickling in transgenic mice than Hb S.

Several studies have indicated that dominant control regions containing erythroid specific DNase I hypersensitive sites that flank the human β -globin cluster play a pivotal role in determining the level of expression of globin genes in erythroid cells (7–12). The ability to link these erythroid dominant control regions to globin genes in transgenic mice has enabled several investigators to achieve high level expression of human globin genes in mouse red cells. Recently, Curtin et al. (12) have cloned a small DNA fragment that spans one of these regions, located 54 kb upstream of the β -globin gene. Linkage of this DNA fragment, designated erythroid specific hypersensitive site II (HS II), to the human β -globin gene results in high level erythroid specific expression of human β -globin in transgenic mice (12).

To maximize the likelihood of creating transgenic mice with red cells that sickle in vivo, we cloned the human β^S Antilles gene and coinjected it with the human α 2-globin gene, each

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1. Abbreviations used in this paper: Hb S Antilles, hemoglobin S Antilles; HS^{II}, hypersensitive site II; ISC, irreversibly sickled cells.

linked to HS II, into fertilized mouse eggs. Mice expressing both human genes were derived. The transgenic mouse red cells contain hybrid mouse α /human β^S Antilles, hybrid human α /mouse β , authentic human S Antilles, and authentic mouse hemoglobin molecules. To raise the ratio of human β^S Antilles to mouse β -globin chains we crossed the human α and β^S Antilles transgenes into the β -thalassemic mouse background. Approximately half the β -globin chains in red cells from the resulting transgenic/ β -thalassemic mice were β^S Antilles. Deoxygenation of the transgenic/ β -thalassemic red cells resulted in significant sickling in vitro, and placement of the animals in an hypoxic environment increased in vivo sickling.

Methods

DNA constructs. A size-selected Hind III library prepared from DNA of an individual heterozygous for the β^S Antilles-allele (5) was constructed in the bacteriophage Charon 30 (13). A human β -globin probe (1.9-kb Bam HI genomic fragment) was used to screen the library (14) and the β -globin inserts from the identified lambda clones were subcloned in pUC18. Normal β and mutant β^S Antilles-alleles were first distinguished by the absence of the codon 6 Mst II site in the latter (15). To further characterize the β -globin clones four allele-specific 19-bp oligonucleotides (β^S Antilles-codon 6 Glu \rightarrow Val mutation: 5' TGACTCTGTGG-AGAAGTC 3', β^S Antilles-codon 23 Val \rightarrow Ile mutation: 5' GTGGATGAAATTGGTGGTG 3', normal β -codon 6: 5' TGACTCTGAGGAGAAGTC 3', normal β -codon 23: 5' GTGGATGAA-GTTGGTGGTG 3') were used as probes. After 32 P end labeling (16) the various allele-specific oligonucleotides were individually hybridized to the 7.8-kb Hind III β -globin insert separated from vector sequences on a 1% agarose gel as described by (17).

The β^S Antilles-construct injected into fertilized mouse eggs consisted of a 5.0-kb Bgl II fragment containing the β^S Antilles gene ligated adjacent to an 800-bp DNA fragment containing HS II (12). The α -globin construct that was coinjected with the β^S Antilles gene was composed of a 1.5-kb Pst I fragment containing the human α 2-globin gene (18) adjacent to HS II (Fig. 2).

Production of transgenic mice. The human α - and β^S Antilles-globin gene constructs were separated from cloning vector sequences by agarose gel electrophoresis and electroelution. The fragments were further purified by passage through an Elutip D column (Schleicher and Schuell, Inc., Keene, NH) followed by ethanol precipitation. A 2- μ g/ml DNA solution containing an equal molar ratio of the α - and β^S Antilles-globin genes was injected into the male pronuclei of Swiss Webster fertilized eggs (Bantin and Kingman, Inc., Fremont, CA). Transgenic founder animals and their transgenic offspring were identified by Southern blot analysis of tail DNA as described by Hogan et al. (19). The human α -probe was a 1.5-kb Pst I fragment containing the entire α -globin gene while the human β -probe was a 1.9-kb Bam HI fragment that includes the 5' end of the human β -globin gene.

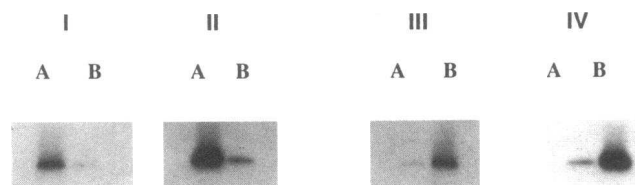


Figure 1. Allele-specific oligonucleotide hybridizations. β^S Antilles-clone, A, and a normal β clone, B, were hybridized to the allele-specific 19-bp oligonucleotide probes for the (I) β^S Antilles-codon 6 Glu \rightarrow Val mutation; (II) β^S Antilles-codon 23 Val \rightarrow Ile mutation; (III) normal β -codon 6; and (IV) normal β -codon 23.

Genetic crosses. The homozygous β -thalassemic mice (Hbb^{th-1}/Hbb^{th-1}) (20) were the kind gift of Dr. Raymond Popp at the Oak Ridge National Laboratory, Oak Ridge, TN. Mice homozygous for the Hbb^{th-1} allele and transgenic for the human α - and β^S Antilles genes (transgenic/ β -thalassemic) were generated by crossing transgenic animals with Hbb^{th-1}/Hbb^{th-1} mice, selecting the transgenic offspring, and backcrossing with Hbb^{th-1}/Hbb^{th-1} mice. The Hbb^{th-1}/Hbb^{th-1} mice were identified by the presence of β^{min} and the absence of β^{maj} mouse β -globin chains by reverse-phase HPLC.

Globin chain analysis. The globin chains and their tryptic fragments were separated by reverse-phase HPLC. Hemoglobin was run on a C-4 (1 \times 25 cm, 300 Å pore, 5- μ m particle size) column (Vydac, Hesperia, CA) at a flow rate of 1.7 ml/min. A modified nonlinear gradient was used to develop the column (21). Tryptic fragments were prepared from the isolated human globin chains and separated by reverse-phase HPLC (22). Evaporated HPLC fractions were analyzed in a glycerol:thioglycerol (1:1) 0.1 N HCl matrix by liquid secondary ion mass spectrometry on a VG 30-250 quadrupole mass spectrometer (VG Biotech Ltd., Altrincham, UK). The average molecular mass analysis of the intact human α - and β^S Antilles-globin chains was determined by electrospray mass spectrometry (23) on a VG Bio-Q mass spectrometer. Mouse globin chains were identified by Triton urea gel electrophoresis of isolated globin chains (24) compared with known controls. The analytical separation of globin chains isolated from transgenic mouse red cells used a Vydac C4 column (0.46 \times 25 cm) at a flow rate of 1 ml/min.

Hemoglobin analysis. The hemoglobins present in transgenic/ β -thalassemic red cells were separated by ion exchange HPLC (25) on a Synchropak CM 300 (0.46 \times 10 cm) column (SynChrom, Inc., Lafayette, IN) or a TSK CM-5 PW column (0.75 \times 7.5 cm, 10- μ m particle size) (Toyo Soda Manufacturing Co., Ltd., Tokyo, Japan). The composition of each hemoglobin species was determined by first isolating the hemoglobin and then determining its globin chain composition by reverse phase HPLC.

Deoxygenation of red cells. Deoxygenation was performed by blowing humidified nitrogen through a test tube containing 10 μ l of fresh mouse blood in 1 ml of PBS at 4°C for 40 min. The sealed deoxygenated sample was then placed at 37°C for 40 min followed by fixation with 100 μ l of a deoxygenated 1.5% glutaraldehyde solution. Reoxygenation involved uncapping the sealed deoxygenated tube just before fixation, swirling the sample to promote contact with air for 5 min, and then adding the glutaraldehyde fixative.

Analysis of mice subjected to hypoxia. Transgenic and control mice were placed in a hypobaric chamber for 10 d. The chamber provided a high altitude environment equivalent to 22,000 ft (0.42 atm) and an FiO_2 of 8.4%. Before and after the 10-d hypoxic exposure blood from individual mice was sampled and analyzed at the ambient FiO_2 of

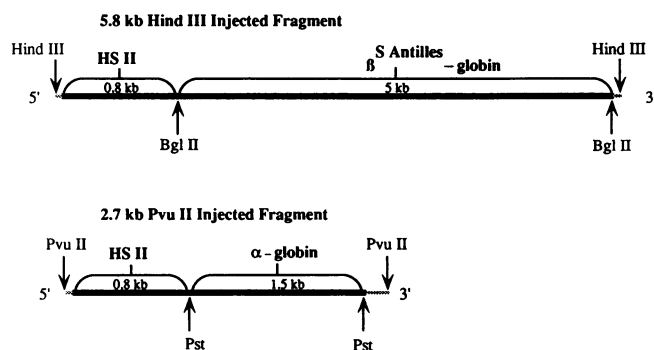


Figure 2. α and β^S Antilles-globin gene constructs. The human α 2-globin gene was used in the α -globin construct while a β^S Antilles-globin gene isolated from an individual heterozygous for this mutation, was used for the β -globin construct. Both globin genes are in the same orientation as the 5' HS II-containing DNA fragment.

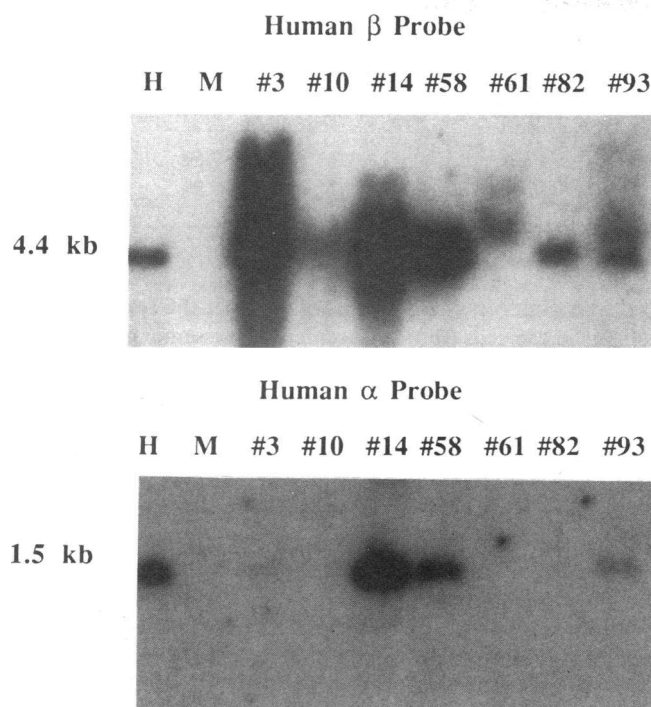


Figure 3. Southern blot analysis of Pst I restriction endonuclease digested DNA from a human control, *H*, a mouse control, *M*, and the seven founder transgenic animals hybridized to human α and β probes. Pst I digestion of human control genomic DNA releases 4.4 and 1.5-kb DNA fragments containing α - and β -globin sequences, respectively.

20.0%. To quantify the volume distribution of red cells before and after hypoxic exposure the volume distribution of red cells from the various animals was analyzed on an H*1 blood cell analyzer (Technicon Instruments Corp., Tarrytown, NY). This instrument measures volume of individual isovolumetrically sphered red blood cells (26). To isolate a population of dense cells for morphological analysis, 50 μ l of blood in a dilute suspension was gently layered above a five step discontinuous Stratan gradient that consisted of 1-ml fractions spanning a density range of 1.074 to 1.120 g/ml in equal increments (27). The filled tube was centrifuged in an SW 27 rotor (Beckman Instruments, Inc., Fullerton, CA) at 20,000 rpm for 30 min at 15°C. Red cells from the most dense bottom layer were isolated fixed with glutaraldehyde and viewed at $\times 400$.

Results

Production of transgenic mice with human α - and β^S Antilles-globin genes. Six β -globin clones were derived from screening of a 500,000 clone recombinant library constructed from the DNA of an individual heterozygous for the β^S Antilles allele (5). The codon 6 mutation in the β^S Antilles gene results in the loss of an Mst II restriction site. Digestion of the six β -globin clones isolated from the library with Mst II indicated that two of the six contained the β^S Antilles allele (data not shown). This result was confirmed by the appropriate hybridization of these clones to oligonucleotide probes specific for the two mutations that distinguish the mutant β^S Antilles allele from the normal β -globin allele (Fig. 1).

The human α and β^S Antilles globin gene constructs that were coinjected into fertilized mouse eggs are shown in Fig. 2. Southern blot analysis of DNA from pups derived from these

Table I. Hematological Indices

	Hematocrit	MCV	MCHC	Reticulocytes
Normal mouse control	47.4 \pm 1.0	48.0 \pm 0.2	33.4 \pm 0.1	2.1 \pm 0.31
Transgenic/ β -thalassemic	45.9 \pm 0.8	44.4 \pm 0.7	33.5 \pm 0.3	2.9 \pm 0.57
β -thalassemic control	28.8 \pm 1.3	39.7 \pm 1.3	29.3 \pm 0.3	10.8 \pm 0.16

injections, using both human α - and β -globin probes, identified seven transgenic founder animals containing human globin genes. Both human α and β^S Antilles sequences were present in three of the mice while the remaining four transgenic animals contained just the β^S Antilles transgene (Fig. 3). Hybridizing the human α -probe to Pst I digests of control human genomic DNA and that of the transgenic founder animals results in a 1.5-kb band. Hybridizing the human β -probe to Pst I digests of the same DNA results in a 4.4-kb band in the human control lane and several bands in lanes containing DNA from the transgenic animals. These hybridization bands that differ between founder animals result from the fact that the injected β^S Antilles-construct contains a single Pst I site 3' to the β^S Antilles coding sequence and 3.8 kb away from the 3' end of the construct. Therefore the size of the released human β^S Antilles-containing fragment can vary depending upon whether the 5' Pst I site is derived from an adjacent human α -globin construct, β^S Antilles construct, or murine genomic DNA in whose midst the β^S Antilles construct integrated. The greater number of β^S Antilles than human α transgenes incorporated into the mouse genome may be explained by the observation that blunt ended DNA fragments, such as the human α -globin construct, are less efficiently integrated into the mouse genome than DNA fragments with cohesive ends, such as the injected β^S Antilles construct (28).

Among the transgenic mice containing both human globin transgenes the highest and near equivalent levels of the human globin chains were present in red cells from founder animals 58 and 14. The following studies were performed on 58 and its offspring. This animal contained approximately 4 copies of the

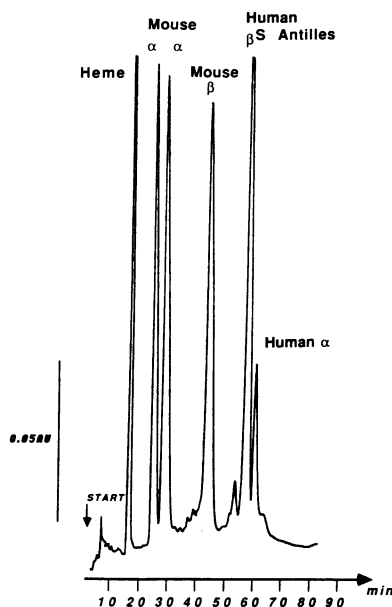


Figure 4. Separation of globin chains of transgenic/ β -thalassemic red cell lysates, by reverse-phase HPLC. The identity of the isolated individual globin chains were determined by a combination of the analysis of their tryptic peptides and comparisons to known controls after Triton urea gel electrophoretic separation.

Table II. Human and Mouse Globin Chain and Hemoglobin Ratios

	α/β	H α /Total α	H β^S Antilles/Total β	H α_2 H β_2^S Antilles Total Hb	M α_2 H β_2^S Antilles Total Hb	H α_2 M β_2 Total Hb	M α_2 M β_2 Total Hb
58 Transgenic founder	1.0	9.0%	13.7%	5.5%	7.8%	7.5%	79.5%
58 Transgenic/ β -thal	1.0	17.3%	49.3%	8.3%	40.4%	5.9%	45.4%

human α and 10 copies of the β^S Antilles gene. Half the offspring of 58 were transgenic and the copy number of the human transgenes was unchanged over three generations, suggesting that the human transgenes integrated at a single locus in the mouse genome in this transgenic line.

Globin chain and hemoglobin analysis. Red cells from founder animal 58 did not sickle upon deoxygenation. Because a higher ratio of β^S - to normal β -globin chains in human red cells increases the likelihood of hemoglobin polymerization and cell sickling, we attempted to raise the ratio of the human β^S Antilles- to murine β -globin chains in the transgenic mouse red cells by crossing the human transgenes from founder animal 58 into a homozygous β -thalassemic mouse background. Homozygous β -thalassemic mice have a deletion of two of the four adult β -globin genes and thus have reduced synthesis of mouse β -globin chains (α to β ratio of 1:3) (20). Animals designated transgenic/ β -thalassemic are hemizygous for the transgene locus and homozygous for the mouse β -thalassemic locus. Normalization of the hematological indices (Table I) and the α to β ratio (Table II) of transgenic/ β -thalassemic mice indicates that expression of the human transgenes corrects the inherent globin chain imbalance and associated anemia of homozygous β -thalassemic mice. These results are consistent with a previous study in which β -globin transgenes alone corrected the thalassemic phenotype when introduced into the homozygous β -thalassemic mouse background (29).

The globin chains in red cells from the transgenic/ β -thalassemic animals were separated by reverse-phase HPLC (Fig. 4). There are two mouse α -globin chains and a single mouse β -globin chain in these red cells in addition to the human α and β^S Antilles-globin chains. The human β^S Antilles-globin chain comprises approximately half the total β -globin chains, while the

human α -globin chain comprises 17% of the total α -globin chains in red cells from transgenic/ β -thalassemic mice. This is a significant increase over the human β^S Antilles-globin chain (14%) and α -globin chain (9%) levels of the founder animal 58 (Table II). The relative increase of β^S Antilles compared with human α -globin chains after transfer of the human transgenes into the β -thalassemic background may be related to the previously demonstrated increased translational efficiency of β - versus α -globin mRNA in murine β -thalassemic red cells (30).

Identification of the human globin chains separated by reverse-phase HPLC was confirmed by preparing tryptic digests of the isolated chains, separating the resulting tryptic peptides and determining their masses by liquid secondary ion mass spectrometry (22). Tryptic digestion of the β^S Antilles-globin chain isolated from the transgenic mouse red cells resulted in two peptides that differed in molecular mass from their normal human β -globin chain counterparts (Fig. 5). Tryptic peptide 1 (T1) with a protonated molecular ion mass of 922 D matches the predicted mass of the human β -globin chain tryptic peptide T1 but with a valine instead of a glutamic acid at position 6. Tryptic peptide 3 (T3) with a protonated molecular ion mass of 1,328 D matches the predicted mass of the human β -globin chain tryptic peptide T3 but with an isoleucine instead of a valine at position 23. The second tryptic peptide in Fig. 5 B,

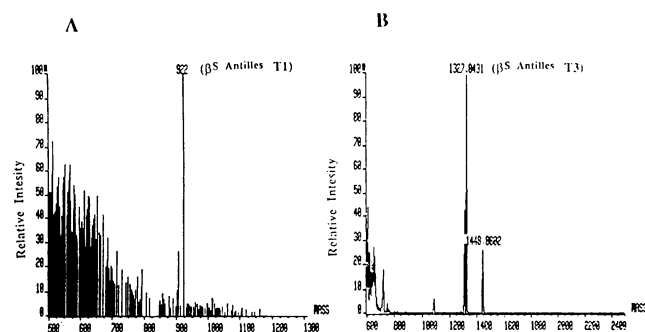


Figure 5. Liquid secondary ion mass spectrometry of the β^S Antilles-globin chain tryptic peptides which differ from that of the normal human β -globin chain. (A) Tryptic peptide 1 with a protonated molecular mass of 922 D as predicted for this tryptic peptide containing a valine at position 6. (B) Tryptic peptide 3 with a protonated molecular mass of 1,328 D as predicted for this tryptic peptide containing an isoleucine at position 23.

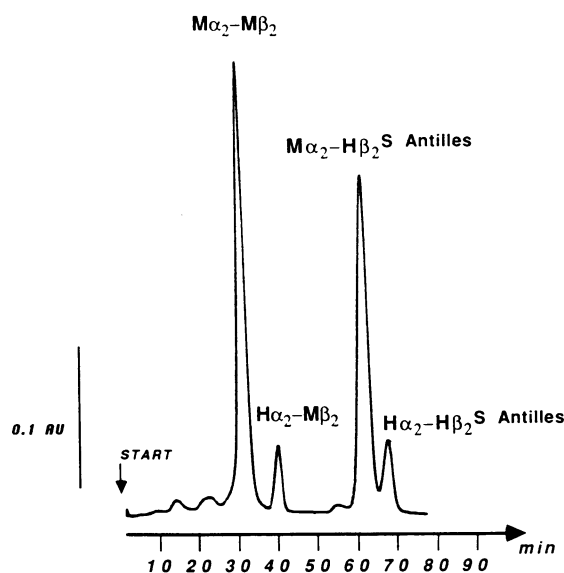
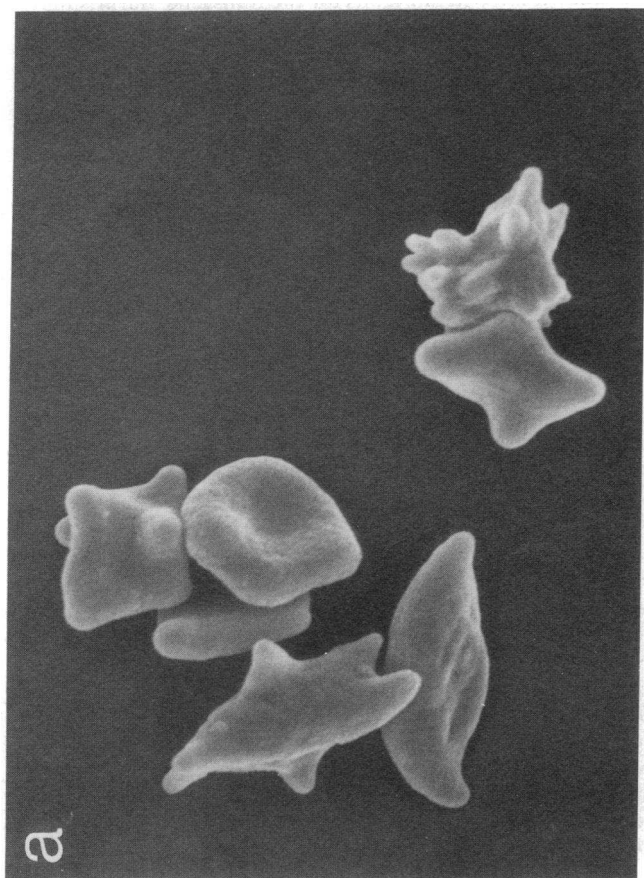
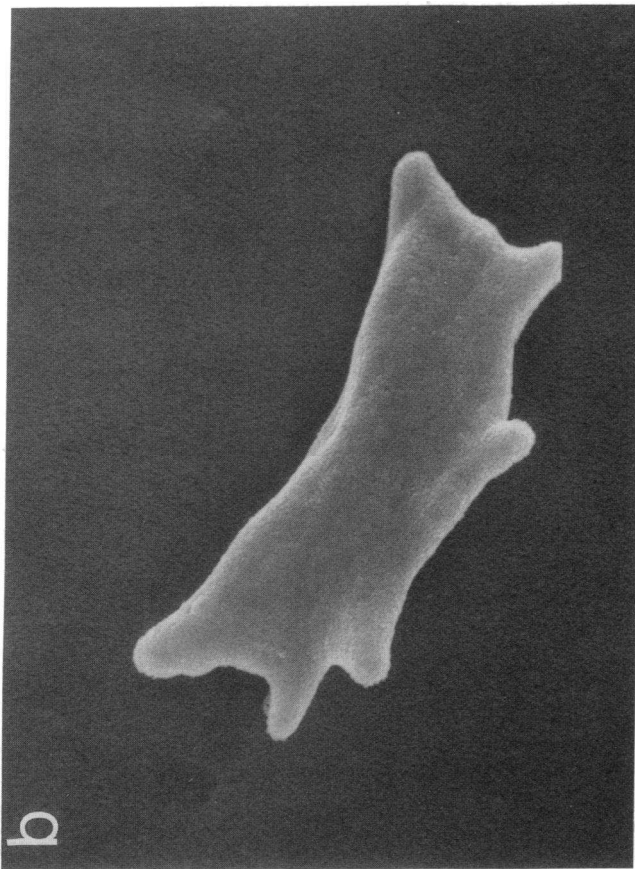


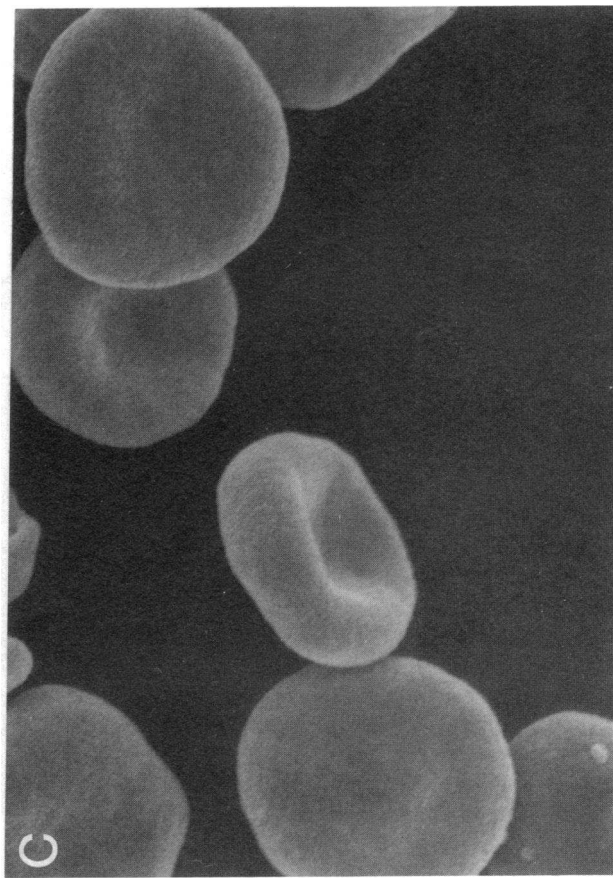
Figure 6. Separation of hemoglobins of transgenic/ β -thalassemic red cell lysates by ion exchange HPLC. The globin chain composition of each hemoglobin species was determined by isolating individual hemoglobin species by ion exchange HPLC followed by reverse-phase HPLC to determine the globin chain composition.



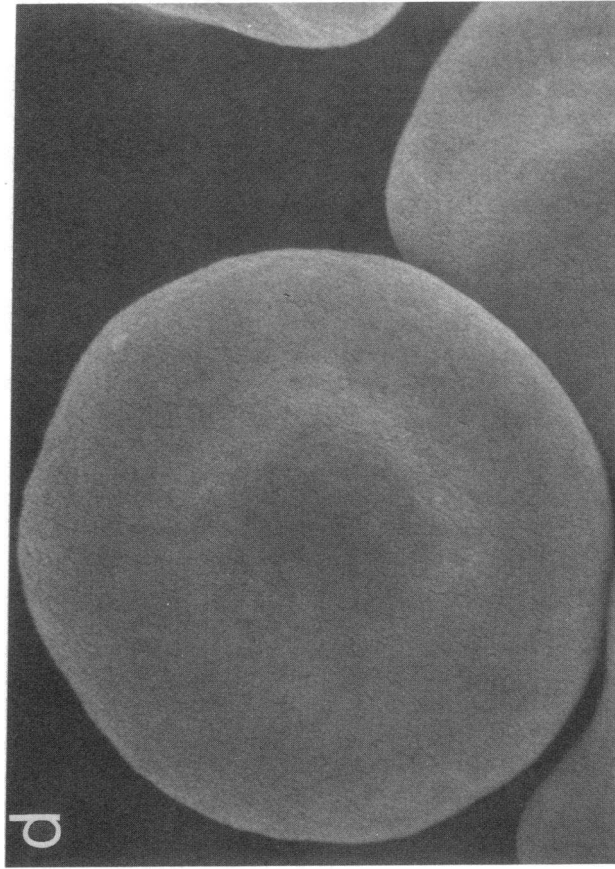
a



b



c



d

Figure 7. Scanning electron micrographs of deoxygenated red cells and red cells that had been deoxygenated and then reoxygenated from a transgenic/ β -thalassemic mouse. (a) and (b) are deoxygenated red cells while (c) and (d) are red cells that were first deoxygenated and then reoxygenated just before fixation. The photographs are $\times 10,000$ to $15,000$.

with a protonated molecular ion mass of 1,448 D represents the β -globin chain tryptic peptide T 14–15 that comigrates on reverse-phase HPLC with the β^S Antilles tryptic peptide T3. The masses of the tryptic peptides of the human α -globin chain isolated from transgenic mouse red cells were identical to a control α -globin sample isolated from human blood (data not shown). The authenticity of the human α - and β^S Antilles-globin chains in mouse red cells was further confirmed by measurements of the molecular masses of intact chains. Measured by electrospray mass spectrometry (23) the average molecular masses of β^S Antilles (15,850.4 D) and human α (15,127.9 D) were within ± 1.5 D of predicted values.

The hemoglobins present in transgenic/ β -thalassemic red cells were separated by ion exchange HPLC (Fig. 6). The hybrid mouse α /human β^S Antilles hemoglobin (40%) along with authentic mouse hemoglobin (46%) are the two predominant hemoglobin species (Table II). The minor hemoglobin species present in these red cells include authentic human Hb S Antilles (8%), and a hybrid human α /mouse β hemoglobin (6%). Red cells from the founder animal 58 have a similar level of authentic Hb S Antilles (6%) but a reduced level of hybrid mouse α /human β^S Antilles hemoglobin (8%) compared with cells from transgenic/ β -thalassemic mice (Table II).

Cellular properties. Classical morphologic sickling was observed after deoxygenation in vitro in $\sim 30\%$ of the transgenic/ β -thalassemic red cells, while the remaining cells exhibited varying degrees of shape abnormalities (Fig. 7, *a* and *b*). These morphological changes were reversed by reoxygenation of the sample (Fig. 7, *c* and *d*). Deoxygenation of nontransgenic thalassemic red cells or red cells from normal mice did not result in sickling or morphological changes (data not shown).

The possibility of in vivo sickling was suggested by the appearance of occasional irreversibly sickled cells (ISCs) in peripheral blood smears. To determine if hypoxia would increase the number of sickled cells formed in vivo we placed the transgenic/ β -thalassemic animals in an hypoxic environment. Since a discernible effect of hypoxia on sickling red cells is a change in cell volume, we quantitated cell volume distribution before and after the hypoxic exposure. The volume of sickled red cells is altered following repeated cycles of hemoglobin polymerization, and red cells from individuals with sickle cell disease exhibit a broader than normal volume distribution. This is believed to be the result of an increased number of cells with smaller volumes due to dehydration and larger volumes due to stress erythropoiesis (26).

The volume distribution of red cells from the transgenic and control animals are similar at an ambient FiO_2 of 20% (Fig. 8). The normal volume distribution curves of red cells from transgenic/ β -thalassemic mice are in agreement with the normalization of the hematological indices (Table I) and the α/β ratio (Table II) of cells from these transgenic mice compared with red cells from nontransgenic β -thalassemic mice. Nontransgenic β -thalassemic red cells have a markedly broadened volume distribution curve (31) that remains largely unchanged after hypoxic exposure (data not shown). After 10 d at an FiO_2 of 8.4% the volume distribution curve of the transgenic but not that of the normal control mice, broadens. This broadening reflects an increased number of cells with both increased and decreased volumes.

Many ISCs and dense fragmented cells, believed to result from repeated cycles of sickling and unsickling in vivo, are

found in the dense cell fraction of red cells from humans with sickle cell anemia (32). To examine red cells from hypoxic transgenic mice with decreased volume and increased density, we isolated dense cells from four transgenic and four control animals using a Stractan gradient. Several typical fields of dense cells from a transgenic animal after hypoxic exposure are shown in Fig. 9. Consistent with the volume distribution curves, nearly every field of dense red cells from the hypoxic transgenic mice contains several ISCs while, before the hypoxic exposure, the most dense fraction of red cells from these transgenic mice had very few ISCs. No ISCs were seen in control mice before or after hypoxic exposure.

Discussion

In this study we have created transgenic mice containing human α - and β^S Antilles-globin genes. Expression of the transgenes results in red cells that sickle upon deoxygenation both in vitro and in vivo, a central feature of sickle cell disease. Our results represent the demonstration of a measurable induction of red cell sickling in an animal in response to hypoxia.

The transgenic animals in our study were homozygous for the mouse β -thalassemic allele in the mouse β -globin cluster on chromosome 7 and hemizygous for the human globin transgene at a separate locus in the mouse genome. We have recently bred mice homozygous for the human α and β^S Antilles transgene containing locus as well as the mouse β -thalassemic allele. Animals were judged as being homozygous for the transgene if all their offspring inherited the transgene coupled with Southern blot analysis demonstrating that their transgene-associated hybridization bands were twice as intense as that of animals known to be hemizygous for the transgene. Surprisingly, no significant increase in the ratio of human α and β^S Antilles to the total α and β -globin chains in red cells from these animals was noted. We are now breeding these doubly homozygous animals to generate a renewable source of genetically defined mice for in vivo sickling studies.

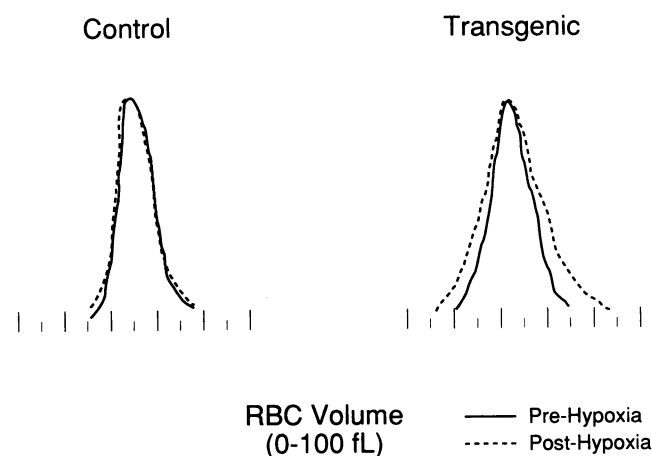


Figure 8. Volume distribution curves of red cells from normal and transgenic/ β -thalassemic mice before and after a 10-d hypoxic exposure. These distributions of individual cells were determined by collecting blood samples before and immediately after the hypoxic exposure and determining the volume of individual isovolumetrically sphered RBCs on a Technicon H*1 flow cytometer.

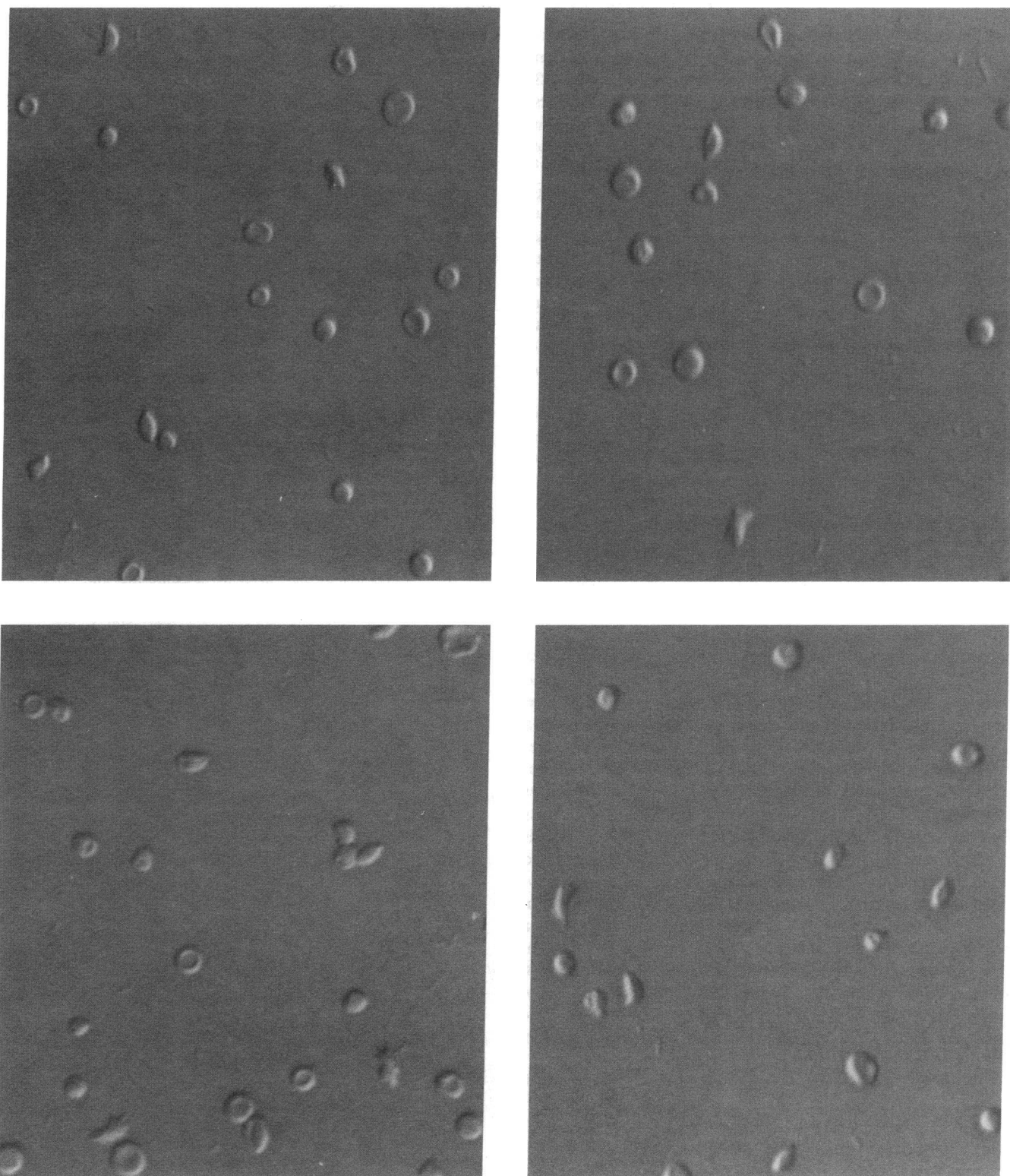


Figure 9. Nomarsky photomicrographs, $\times 400$, of dense cells from a transgenic/ β -thalassemic mouse immediately after 10 d of hypoxia. These red cells were isolated from the most dense bottom layer of a Stratan gradient spanning the density range of 1.074–1.120 g/ml in equal increments and viewed as wet mounts.

The hemoglobins present in the red cells of the transgenic mice in this study were characterized using the combination of ion exchange HPLC, which separates all the hemoglobin species present in these red cells, followed by reverse-phase HPLC

to identify the globin chain composition of each hemoglobin. The predominance of the hybrid mouse α /human β^S Antilles hemoglobin in sickling transgenic red cells suggests that this molecule may be participating in hemoglobin polymerization. This

is further supported by the observation that both in vitro and in vivo sickling occurs in transgenic/ β -thalassemic red cells containing 8% Hb S Antilles and 40% hybrid mouse α /human β^S Antilles hemoglobin, but does not occur in red cells of the 58 founder animal that contain a similar amount of Hb S Antilles (6%) but significantly less of the hybrid mouse α /human β^S Antilles hemoglobin molecule (8%). Prior in vitro analyses of the kinetics of polymerization of hybrid mouse α /human β^S Antilles as well as hybrid mouse α /human β^S hemoglobin suggested that these mouse/human hybrids would not polymerize (6). However, it should be noted that the Hb concentrations used in these earlier studies were 250-fold lower than that found in red cells. This could explain the discrepancy between our findings with intact red cells and the previous solution assays. In two other studies that examined sickling of transgenic red cells containing Hb S (2, 3), the contribution of the hybrid mouse α /human β^S molecule to total red cell hemoglobin was not determined.

Of the studies examining the effects of Hb S on transgenic red cells (2, 3), a mouse with the highest levels of human Hb S, 84%, was described by Greaves et al. (2). Although significant in vitro sickling of red cells from this animal was observed, other than the observation of $\sim 0.1\%$ irreversibly sickled cells in peripheral blood smears, normal somatic development and absence of hemolytic anemia suggest that despite the predominance of Hb S this animal experienced minimal in vivo sickling. Red cells from another transgenic mouse in this same study, where the ratio of Hb S to total hemoglobin was 35%, did not sickle upon complete deoxygenation in vitro. In a separate study also examining the effect of Hb S on transgenic mouse red cells Ryan et al. (3) characterized two lines of transgenic mice where the ratio of Hb S to total hemoglobin was 50%. In one line of animals the human α and β^S transgenes were in a non- β -thalassemic mouse background while in the second series of animals these transgenes had been moved into a heterozygous mouse β -thalassemic background. Despite differences of the mouse backgrounds red cells from both series of animals were reported to contain 50% Hb S. Less than 1.0% of red cells from the transgenic non- β -thalassemic mice sickle in vitro while greater than 90% of red cells from the transgenic heterozygous β -thalassemic mice sickle upon complete deoxygenation in vitro. In contrast to the observations of Greaves et al. (2) on a transgenic mouse containing 84% Hb S, the transgenic β -thalassemic mice containing 50% Hb S described by Ryan et al. (3) developed mild anemia, reticulocytosis, and splenomegaly. The differing results of these two studies make it difficult to draw conclusions concerning in vivo consequences of Hb S in mouse red cells.

With regard to the in vivo consequences of mouse red cells containing human α and β^S Antilles globin chains we have been able to conclude in our study that in vivo sickling increases in animals during hypoxia. The transgenic mice we described contain considerably fewer α -globin chains in their red cells than the transgenic animals described by Greaves et al. (2) and Ryan et al. (3). This observation, coupled with the lack of information concerning individual globin chain or hybrid hemoglobin levels in the two Hb S transgenic mouse studies (2, 3), makes it difficult to compare in vivo properties of human α and β^S -containing red cells to that of human α and β^S Antilles-containing cells. The significant in vitro sickling and an inducible in vivo sickling of red cells containing 50% β^S Antilles globin

chains but only 8% Hb S Antilles does support our prediction concerning the efficacy of Hb S Antilles and β^S Antilles globin chains to foster sickling of mouse red cells both in vitro and in vivo.

The ability to detect and quantitate red cell changes in response to hypoxia in transgenic mice may provide new opportunities for examining red cell sickling in vivo. This system could be used to perform studies that are difficult to do in humans, such as examining the efficacy of experimental antisickling agents. In spite of differences between Hb S and Hb S Antilles, the need for an animal model for sickle cell anemia, the lack of which has long hindered the study of this disease, may in part be met by this transgenic mouse model.

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