The βIVS-2-654 C→T mutation accounts for approximately 20% of β thalassemia mutations in southern China; it causes aberrant RNA splicing and leads to β-thalassemia. To provide an animal model for testing therapies for correcting splicing defects, we have used the “plug and socket” method of gene targeting in murine embryonic stem cells to replace the two (cis) murine adult β globin genes with a single copy of the human βIVS-2-654 gene. No homozygous mice survive postnatally. Heterozygous mice carrying this mutant gene produce reduced amounts of the mouse β globin chains and no human β globin, and have a moderate form of β thalassemia. The heterozygotes show the same aberrant splicing as their human counterparts and provide an animal model for testing therapies to correct splicing defects at either the RNA or DNA level.

MATERIALS AND METHODS

Gene targeting. The socket-containing embryonic stem (ES) cell line, B20, in which a neo gene and a partially deleted minigene for hypoxanthine phosphoribosyl transferase (HPRT) are inserted downstream of the murine adult β globin genes, was used to introduce the mutant form of the human gene. The construct, th-4 plug (see Fig 1), includes a 5.7-kb genomic HindIII-XhoI fragment of the human β globin gene, covering the GenBank sequence region 59611-65439, into which the human βIVS-2-654 mutation was introduced by site-directed mutagenesis (see Fig 1). The th-4 targeting construct also contains a 3.9-kb BamHI-HindIII fragment of BALB/c mouse DNA inserted 5′ to the murine adult β globin genes and inserted 3′ to the mouse genes, a 1.9-kb ClaI-XhoI fragment, which contains the promoter, and exon 1 of the HPRT minigene, as described previously. The β globin and HPRT genes are in the same transcriptional orientation. Th-4 targeting DNA was introduced into B20 cells by electroporation and hypoxanthine-aminopterin-thymidine (HAT)-resistant, G418-sensitive colonies were isolated. The presence on Southern blots of a 10-kb NdeI fragment hybridizing to a probe specific to intron 2 of the human β globin gene was used to confirm correctly targeted colonies.

Chimera production and breeding. Three germine transmitting chimeras were generated from one of three targeted ES cell lines that were isolated. Chimeras were bred to C57BL/6J (B6) mice to produce F1 (129×B6) offspring. The F1 animals used for the studies described here are genetically identical except for the presence (Hbb0-Hbb+) or absence (Hbb0-Hbb−) of the human βIVS-2-654 gene at the β globin locus.

Cellulose acetate electrophoresis. For hemoglobin analysis by cellulose acetate electrophoresis, heparinized blood samples were collected from the retroorbital sinus and washed twice in 20× volumes of buffered saline to isolate red blood cells (RBC). The RBC were lysed in 50× volumes of cold deionized, distilled water. A total of 10 µL of lysate was then mixed with 2 µL of 75 mg/mL cystamine dihydrochloride (pH > 7) to modify the hemoglobin sulfhydryl groups. After incubation for 10 minutes at room temperature, approximately 3 µL of the modified lysate was analyzed by electrophoresis on Titan III-H cellulose acetate strips (76 × 60 mm) (Helena Laboratories, Beaumont, TX). Electrophoresis was performed with Supre-Heme buffer pH 8.2 (Helena Laboratories) at 40 V/cm. Bands were stained with 0.5% Ponceau S and destained with 5% acetic acid.
Peripheral blood smears. Peripheral blood smears, made from 1 to 2 μL of blood collected in heparinized microhematocrit tubes, were air dried and stained with Wright stain.

RBC indices. Whole blood samples from mice at least 8 weeks old were collected in 40 μL microhematocrit tubes containing 2 μL of 0.5 mol/L EDTA (pH 8). The hematocrit (Hct), RBC count, hemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and RBC distribution width (RDW) for each sample was determined using a Roche Cobras Helios Hematology Analyzer (ABX, Montpellier, France) equipped with software to analyze murine cells.

Organs weights and preparation. Mice at 5 months of age were given lethal doses of 2.22 tribromoethanol (avertin) and perfused with 4% paraformaldehyde (pH 7.4). Liver, lungs, heart, spleen, and kidneys were collected from each animal, kept overnight in Bouin's solution, blotted dry, weighed, embedded in paraffin, and sectioned. Staining was with hematoxylin and eosin or Prussian blue.

RNA isolation. Blood was collected from mouse tail veins in microhematocrit tubes previously rinsed with sterile acid citrate dextrose. Total RNA was prepared using the Tri-Reagent BD system (Molecular Research Center, Cincinnati, OH) as described by the manufacturer.

Reverse transcriptase-polymerase chain reaction. Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed on the RNA with rThi enzyme (Perkin Elmer, Foster City, CA) with 32P-labeled nucleotide triphosphates for 18 cycles of 95°C 1 minute and 65°C for 1 minute. PCR products were separated on a 7.5% nondenaturing polyacrylamide gel.

The primers (see Fig 3A) used to determine aberrant splicing of the human β globin pre-mRNA were: i, 5'-GAGGCA TGA TACA TTGTA TCA TTA TTGCCCC-3', and ii, 5'-GCACAAGGCTTCTT GAGTCC-3', which correspond respectively to nucleotides 21-43 of the second exon and nucleotides 28-6 of the third exon. To identify the region of intron remaining in the processed human mRNA, the 5' primer i was used with primer iii, 5'-GAGGCA TGA TACA TTGTA TCA TTA TTGCCCC-3', corresponding to 2 bases of sequence at the end of exon 2 and 29 bases of intron 2.

RESULTS

The “plug and socket” method of targeting is a two-step procedure. The first step was applied to the murine β globin locus to produce a generally useful “socket”-containing embryonic stem (ES) cell line (B20), which has been described. 5 The B20 cell line, derived from an HPRT-deficient ES cell line, contains a neomycin resistance (neo) gene and a partial HPRT gene (ΔHPRT) downstream of the murine β major and β minor globin genes (Fig 1A). For the second step, which generates the desired mutation, a “plug” targeting construct (Fig 1B) was used that allowed the replacement of the 21-kb of mouse genomic DNA containing the murine β major and β minor globin genes with a 5.7-kb DNA fragment containing the human βIVS-2-654 gene. The resultant mutation (Fig 1C) is designated Hbbth-4 (abbreviated to th-4).

To determine if the human β globin pre-mRNA produced in the Hbbth-4/Hbb+ mice was aberrantly spliced as in humans, RT-PCR was performed on RNA derived from whole blood of a Hbbth-4/Hbb+ mouse, a mouse (huβ/Hbb+) heterozygous for a chromosome carrying a single copy of the human sickle globin gene in place of the murine adult β globin genes (Lewis et al, unpublished), and a normal human. Complementary primers to human (but not mouse) β globin exons 2 and 3 were used to amplify a 230-bp region in correctly spliced human β globin mRNA or a 303-bp region in aberrantly spliced βIVS-2-654 mRNA. Only correct splicing of the human β globin mRNA was detected in the huβ/Hbb+ mouse and in the normal human (Fig 2B, lanes 1 and 4). Only aberrantly spliced β globin mRNA was detected in the heterozygous β thalassemic mice (Fig 2B, lane 2). RNA from a HeLa cell line transfected with a human β globin gene containing the IVS-2-654 mutation6 was used to identify the correct size of the aberrantly spliced β globin

Fig 1. Replacement of the murine adult β globin genes by the human βIVS-2-654 gene. The socket-containing chromosome (A), the th-4 plug targeting construct (B), and the correctly targeted chromosome (C) are shown. The exons and introns of genes are represented as boxes and thick lines, respectively. The human β globin gene is cross-hatched with the position of the IVS-2-654 mutation shown with an asterisk. Promoter (P) and exons 1-9 of HPRT are marked. Upstream and downstream sequences that are identical or homologous in the targeting construct and the target chromosome are demarcated by dashed lines. βh3 is a β globin pseudogene. Recombination (indicated by Xs) occurs between the target locus (A) and the plug targeting construct (B), yielding a chromosome that contains the human β globin gene in place of the adult murine β globin genes, β major and β minor. Additionally, the neo gene is removed and a functional HPRT gene is created by the correct targeting. The HPRT gene and globin genes are transcribed from left to right in the figure, the neo gene is transcribed from right to left.
transcript (Fig 2B, lane 3). No amplification was observed with RNA from wild-type mice (data not shown).

When the 5’ primer i was used in combination with a 3’ primer, iii, which includes two bases from exon 2 and continues into the intronic region that is only retained in aberrantly spliced \( \beta \)IVS-2-654 mRNA, the \( \beta \)IVS-2-654 thalassemic mouse and the transfected HeLa cell line showed the expected 233-bp band (Fig 3B, lanes 2 and 3). The heterozygous hu\( \beta \)s/\( \beta \)1 mouse and the normal human showed no amplification product (Fig 3B, lanes 1 and 4). These results show that the \( \beta \) globin pre-mRNA produced in the \( \beta \)IVS-2-654 mice was aberrantly spliced using the same IVS-2-579 and IVS-2-652 splice sites as are used in humans with the IVS-2-654 mutation.

Heterozygous mice (\( \beta \)IVS-2-654/\( \beta \)1), where \( \beta \)1 represents the wild-type \( \beta \) globin locus from strain C57BL/6J, were noticeably smaller and paler than their \( \beta \)1/\( \beta \)1 littermates at

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**Fig 2.** A diagram and RT-PCR results showing aberrant splicing of the human \( \beta \)IVS-2-654 transcript in the \( \beta \)IVS-2-654 thalassemic mice. (A) The diagram shows the human \( \beta \)IVS-2-654 gene with aberrantly spliced \( \beta \) globin mRNA produced from the mutant gene compared with the correctly spliced \( \beta \) globin mRNA that would have been produced from a wild-type gene. The thick line between nucleotides 580 and 652 shows the region of IVS-2 that is maintained in the \( \beta \)IVS-2-654 mRNA. RT-PCR primers i and ii are shown at the location and in the direction in which they anneal to the RNA. Primer i anneals to sequences within the second exon of human \( \beta \) globin, and primer ii anneals to sequences within the third exon of human \( \beta \) globin. (B) An autoradiograph of polyacrylamide gel electrophoresis of RT-PCR with primers i and ii on RNA from a heterozygous hu\( \beta \)s/\( \beta \)1 mouse (lane 1), a heterozygous \( \beta \)IVS-2-654/\( \beta \)1 thalassemic mouse (lane 2), a HeLa cell line transfected with a \( \beta \)IVS-2-654 gene (lane 3), and a normal human (lane 4) is shown. Aberrantly spliced \( \beta \) globin mRNA RT-PCR products are 303 bp and 230 bp, as labeled.

**Fig 3.** A diagram and RT-PCR results showing aberrant splicing as seen in humans of the human \( \beta \)IVS-2-654 transcript in the \( \beta \)IVS-2-654 thalassemic mice. (A) The diagram shows the human \( \beta \)IVS-2-654 gene with aberrantly spliced \( \beta \) globin mRNA produced from the mutant gene compared with the correctly spliced \( \beta \) globin mRNA that would have been produced from a wild-type gene. The thick line between nucleotides 580 and 652 shows the region of IVS-2 that is maintained in the \( \beta \)IVS-2-654 mRNA. RT-PCR primer i and ii are shown at the location and in the direction in which they anneal to the RNA. Primer i anneals to sequences within the second exon of human \( \beta \) globin, and primer ii anneals to sequences within the third exon of human \( \beta \) globin. (B) An autoradiograph of polyacrylamide gel electrophoresis of RT-PCR with primers i and ii on RNA from a heterozygous hu\( \beta \)s/\( \beta \)1 mouse (lane 1), a heterozygous \( \beta \)IVS-2-654/\( \beta \)1 thalassemic mouse (lane 2), a HeLa cell line transfected with a \( \beta \)IVS-2-654 gene (lane 3), and a normal human (lane 4) is shown. Aberrantly spliced \( \beta \) globin mRNA RT-PCR product is 233-bp, as labeled. Correctly spliced \( \beta \) globin mRNA does not amplify with primers i and ii.
Hematologic values are expressed as means ± SEM. *P values are for two-tailed Student’s t tests in comparisons between Hb°/Hbb° and Hb°-4/Hbb° mice assuming equal variance.

Abbreviations: RBC, red blood cells; Hb, hemoglobin; Hct, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, RBC distribution width.

* Denotes significant P values.

DISCUSSION

Four types of mouse models for human β thalassemia have been described, including a naturally occurring β thalassemia observed in mice. In the first model, which is a naturally occurring deletion, one of the two mouse adult β globin genes, β major, is deleted. About 60% of mice homozygous for this deletion (Hb°/Hbb°) survive to adulthood. Heterozygotes (Hb°+/Hbb°) show very mild thalassemia. The second model for β thalassemia was created by insertional disruption by gene targeting of the mouse adult β major globin gene. Mice homozygous for this mutation (Hb°/Hbb°) do not survive past a few hours after birth. The heterozygotes are anemic and have features of thalassemia similar to those found in human β thalassemia intermedia. Two models (Hbb°-/Hbb°-15) were produced by complete deletions of both the murine adult β globin genes, β major, and β minor. The phenotypes of the heterozygotes for these two models are equivalent and include birth. No homozygous mutant animals survived postnatally. Cellulose acetate electrophoresis of the hemoglobin from the heterozygous Hbb°-4/Hbb° mice showed no hemoglobin from the human mutant gene (data not shown). The heterozygous Hbb°-4/Hbb° mice showed classic signs of β thalassemia intermedia, including anisocytosis, poikilocytosis, and target cells in the peripheral blood smear (Fig 4). Changes in RBC indices were observed in Hbb°-4/Hbb° mice when compared with their Hbb°+/Hbb° littermates (Table 1), including significant decreases in RBC count, Hb, Hct, MCV, and increases in MCHC and RBC distribution width.

Body weights of F1 heterozygous mice at 2 months of age (21.8 ± 0.9 g, standard error of mean [SEM] n = 7) were 14% smaller than wild-type mice (24.8 ± 1.1 g, n = 7), although the difference did not quite reach statistical significance (P = .052). Lungs, livers, kidneys, and hearts of wild-type and thalassemic animals comprised approximately the same percentages of body weight. However, the spleens of the Hbb°-4/Hbb° mice were dramatically enlarged (P = 1.8 × 10⁻⁴) and comprised 2% of body weight compared with 0.2% of body weight in wild-type mice. This result is consistent with the splenomegaly usually seen in thalassemic patients.

At 2 months of age, the livers from heterozygous Hbb°-4/Hbb° mice showed extramedullary hematopoiesis, similar to that observed in human thalassemia, with dilated sinusoids containing hematopoietic cells. The livers and spleens of Hbb°-4/Hbb° mice contained regions of iron deposition that were increased by 5 months of age. Similar iron deposits are frequently seen in human thalassemia. The hearts, lungs, and kidneys of the 2-month-old animals showed no iron deposition, but by 5 months, the convoluted tubules of the kidneys of the Hbb°-4/Hbb° mice had substantial iron deposition. The Hbb°-4/Hbb° mice are more severely affected than comparable β1/β1 thalassemia intermedia humans, presumably because mice cannot compensate for a shortage of β globin subunits by maintaining production of fetal globins or by increasing δ globin synthesis.

Overall our data establish unequivocally that the human IVS-2-654 gene is transcribed in the Hbb°-4/Hbb° mice and that all of the corresponding processed mRNA is 73-bp larger than that expected from normal processing.
microcytic anemia and splenomegaly. The th-3 homozygotes die immediately after birth.

The present mouse model for β thalassemia is a heterozygote (Hbb th-4/Hbb th-4) carrying a human gene with βIVS-2-654 splice mutation and the normal mouse β globin locus; it shows the classic signs of a moderate form of β thalassemia. The th-4/Hbb th-4 heterozygous mice have low RBC counts, indicating inefficient erythropoiesis and increased RBC destruction. This is seen in humans with β thalassemia and is due to inclusion body (alpha 4 tetramers) precipitation on the membrane of the RBC before they are released into circulation. As expected, peripheral blood smears from the Hbb th-4/Hbb th-4 mice, showing marked anisocytosis and poikilocytosis, are similar to those of the heterozygous Hbb th-3 mice, but are more substantially pronounced than the smears from heterozygous Hbb th-2 mice. The genetic defect clearly results in profound RBC morphologic abnormalities reflective of the associated erythropoietic abnormalities. These morphologic changes are very similar to those observed in human thalassemia. At 2 months, the thalassemic animals had not begun to accumulate iron in their kidneys, but by 5 months, iron deposits could be seen throughout the convoluted tubules of the thalassemic animals, presumably the consequence of ongoing hemolysis and increased iron absorption as seen in human thalassemia.

Thus, the heterozygous Hbb th-4/Hbb th-4 mice exhibit the β thalassemia intermediate phenotype and provide the first animal model of any disease resulting from a known human splicing mutation. In addition, unlike mouse models for thalassemia caused by complete inactivation or deletion of genes in which direct gene therapy requires the addition of a functional gene, the Hbb th-4 animals can be treated in ways designed to correct the aberrant splicing at both the RNA and DNA level. Complete correction is expected to normalize their thalassemic condition because heterozygous mice carrying a human sickle globin gene in the same context as the βIVS-2-654 gene in the Hbb th-4 heterozygotes are not thalassemic (J. Lewis et al, unpublished data). But even a small increase in the production of correctly spliced mRNA should be clearly beneficial in decreasing the severity of the thalassemia and should be detectable without the need to kill the animals by testing for human β globin polypeptide or mRNA in their circulating RBC or reticulocytes. The presently generated mice will therefore provide an animal model in which the antisense and other types of therapy can be tested in vivo.

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REFERENCES

A Common Human β Globin Splicing Mutation Modeled in Mice

Jada Lewis, Baoli Yang, Ronald Kim, Halina Sierakowska, Ryszard Kole, Oliver Smithies and Nobuyo Maeda