Humanized β-Thalassemia Mouse Model Containing the Common IVSI-110 Splicing Mutation*

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Splicing mutations are common causes of β-thalassemia. Some splicing mutations permit normal splicing as well as aberrant splicing, which can give a reduced level of normal β-globin synthesis causing mild disease (thalassemia intermedia). For other mutations, normal splicing is reduced to low levels, and patients are transfusion-dependent when homozygous for the disease. The development of therapies for β-thalassemia will require suitable mouse models for preclinical studies. In this study, we report the generation of a humanized mouse model carrying the common IVSI-110 splicing mutation on a BAC including the human β-globin (hμ-β-globin) locus. We examined heterozygous murine β-globin knock-out mice (μβ-β3/−) carrying either the IVSI-110 or the normal hμ-β-globin locus. Our results show a 90% decrease in hμ-β-globin chain synthesis in the IVSI-110 mouse model compared with the mouse model carrying the normal hμ-β-globin locus. This notable difference is attributed to aberrant splicing. The humanized IVSI-110 mouse model accurately recapitulates the splicing defect found in comparable β-thalassemia patients. This mouse model is available as a platform for testing strategies for the restoration of normal splicing.

Thalassemia is one of the common inherited genetic disorders affecting hemoglobin synthesis. Approximately 300,000 patients with clinically relevant hemoglobinopathies are born each year (1). In the case of β-thalassemia a reduction or absence of β-globin chain synthesis results in free or unpaired α-globin chains, which aggregate and precipitate within red cells causing ineffective erythropoiesis and severe anemia. Over 200 different mutations have been found that cause β-thalassemia, with splicing mutations among the most common. Most of these mutations activate aberrant cryptic 5′-donor or 3′-acceptor splice sites without completely abolishing normal splicing. These mutations lead to the production of variable amounts of normal transcripts. Some mutations allow a significant level of normal splicing (such as IVSI-6), leading to thalassemia intermedia, whereas others reduce normal splicing to low levels (such as IVSI-110) or very low levels (such as IVSI-5 and IVSII-654), causing a transfusion-dependent disease in homozygotes.

Because of the complex pathophysiology associated with the hemoglobinopathies, transgenic mouse models are an essential platform for delineating the pathological mechanisms and, as in vivo model systems, for validating future therapeutic strategies. The humanized mouse model approach, where the transgenic mouse model contains large human genomic fragment(s), has provided valuable insight into the regulatory elements required for the developmental expression of human globin genes (2–6). To date, the YAC transgenic mouse for sickle cell anemia, exclusively expressing human β-globin, is the only mouse thalassemic model expressing a disease gene from the intact hμ-β-globin locus (7). Other transgenic mice exclusively expressing human HbA, HbC, HbF, and HbS have also been reported, but these mouse models were created by co-injecting large genomic DNA fragments encompassing truncated or hybrid versions of the β-globin locus containing the α-globin gene (8–10). The only β-thalassemia splicing mouse model reported to date is a knock-in model that contains the common IVSII-654 β-thalassemia splicing mutation in the context of the mouse β-globin (mu-β-globin) locus (11).

With the recent development of targeted modification techniques for BACs such as recE/recT-based homologous recombination (12–15), and the availability of site-specific BAC mutagenesis (16–19), the main obstacle to generating transgenic mouse models for disease-causing mutations has been overcome. We have recently reported the first humanized mouse model for a deletion mutation causing β-thalassemia at codons 41–42 (20). Unlike the situation with the normal hμ-β-globin locus, these mice fail to show phenotypic complementation in heterozygous β-globin knock-out (KO) background (μβ-β3/−), or homozygous β-globin knock-out mice (μβ-β3/−). This mouse is a suitable in vivo model system to investigate gene correction strategies in hematopoietic stem cells and to identify HbF inducers.

We describe the development and characterization of a humanized mouse model carrying the IVSI-110 splicing mutation in the context of the genomic hμ-β-globin locus. This mutation was first described in 1981 and is one of the most common splicing mutations found in the Eastern Mediterranean region (21). Our results indicate that this humanized IVSI-110 mouse model recapitulates the splicing defect that is typical for IVSI-110 in β-thalassemia patients. This mouse will serve as a platform for testing novel strategies for the restoration of normal splicing.

MATERIALS AND METHODS

Characterization of the 183-kb Genomic Fragment Containing the hμ-β-Globin Locus—The PAC clone 148O22 containing the hμ-β-globin locus in a 183-kb genomic fragment was first isolated from the RPCI I PAC library (22) and shown to contain the β-globin locus (23). The 183-kb genomic fragment was retrofitted into the pEBAC140 cloning vector as a single NotI fragment to generate pEBAC148β (see Fig. 1A) (24). Sequencing of the 5′- and 3′-ends of the genomic insert (data not shown) and alignment with the human genome sequence (GenBank™ accession number NT_028310.10) revealed that the genomic insert is...
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183.039-bp long, with 122,076 bp upstream of the start codon of the e-globin gene and 17,672 bp downstream of the stop codon of the β-globin gene.

Introduction of the IVSI-110 Mutation into the β-Globin Locus—Using GET recombination, an inducible homologous recombination system for Escherichia coli, the IVSI-110 mutation was introduced into the intact β-globin locus contained in a BAC vector (14). The β-globin locus containing the IVSI-110 mutation was purified from DH10B cells using the Qiagen BAC purification procedure (Qiagen, Hilden, Germany). The 183-kb genomic insert was prepared for microinjection as previously reported (25).

Genotyping of Transgenic Mice—Genotyping was performed as described previously (25). In brief, transgenic founder mice and F1 progeny were screened by PCR using the following primer pair, huβ forward 5’-ACAAGACAGGTATTAAGGAGCCA-3’ and huβ reverse 5’-GTCTGTTTCCATTCTAAGCTGA-3’. These primers amplified a 447-bp product of the huβ-globin gene. F1 progeny were bred with heterozygous knock-out mice (huβ−/−) to generate transgenic mice on a heterozygous and knock-out background. The genotyping was performed by multiplex PCR using the following primer pairs: huβ forward/ huβ reverse (as above); HPRT-forward, 5’-GATGGAGGCGCTACAC-ATTGTAG-3’ and HPRT-reverse, 5’-GGGACCTTGACCATCTTTTGGATTA-3’; and μβ-forward, 5’-TGAAGAAAGCTGTCCTTTG-3’ and μβ-reverse, 5’-CAGAGGATAGGTCTCCAAAGCTA-3’.

The conditions used for the multiplex PCR were denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 2 min. After 30 cycles, the PCR products were resolved by 1.5% agarose gel-electrophoresis.

Determination of Transgene Integrity—The long range integrity of the huβ-globin locus in transgenic lines was examined by Southern blot analysis after digestion of genomic DNA with the Cfr91 and SfiI restriction endonucleases (Fermentas, Hanover, MD). Cfr91 digestion of the huβ-globin locus yields a single 36-kb fragment that contains the γ-, δ-, and β-globin genes, whereas SfiI digestion cuts the genomic fragment twice in the middle of the fragment (see Fig. 1). To avoid shearing the genomic DNA, splenocytes from representative transgenic mice were suspended at a final concentration of 2 × 10⁶ cells/ml in 0.5% InCert agarose (Cambrex Bio Science). The solidified plugs were incubated overnight in buffered Proteinase K buffer (100 mM EDTA at pH 8.0, 0.2% sodium deoxycholate, 1% sodium lauryl sarcosine, 0.2 mg/ml of Proteinase K) at 50 °C. After several washes in 20 mM Tris, 50 mM EDTA at pH 8.8, the plugs were digested overnight with Cfr91 or SfiI. The plugs were loaded into the wells of a 1% agarose gel, and genomic DNA was separated in a CHEF-DRII pulsed-field gel electrophoresis system (Bio-Rad). The DNA was alkali-blotted onto a Hybond membrane (Amersham Biosciences) after partial depurination and hybridized with a PCR probe amplified from the huβ-globin locus using primers huβ-forward/huβ-reverse.

Determination of Transgene Copy Number—Transgene copy number in mice was performed by quantitative radioactive PCR as previously reported (25). In brief, PCR was performed using the primer pair HM-forward 5’-GATGAAATTGGTGTTGAGGCCCT-3’/HM-reverse 5’-CCTGAAGTTCTCAGGATCCA-3’ on mouse tail genomic DNA. These primers amplify the huβ-globin and muβ-globin sequences equally, yielding 381-bp and 367-bp products, respectively. The human and mouse products can be readily distinguished because the murine product contains a unique NcoI site that yields two fragments, 242 and 125 bp. The NcoI-digested PCR products were run on a 2.0% 3:1 Nusieve gel (BMA). The gel was washed for 45 min, in 7% trichloroacetic acid and vacuum-dried at 65 °C onto blotting paper. The dried radio-active gel was exposed to a Low Energy Storage Phosphor Screen (Molecular Dynamics, Amersham Biosciences), and the Phosphor Screen was scanned using the Storm 840 Scanner (Molecular Dynamics). Each band corresponding to huβ-globin and μβ-globin PCR product was quantitated using Image Quant (Molecular Dynamics).

Location of Transgene Integration Site—Fluorescence in situ hybridization analysis was used to examine transgene integration sites and confirm hemizygosity or homozygosity for the transgene in mice exclusively expressing huβ-globin. Fibroblasts isolated from tail biopsies, were grown in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. The fibroblasts were harvested after exposure to colcemid for 2 h. Chromosome preparations were obtained using standard techniques and hybridized with a pEBAC/148βDNA probe. The probe was labeled with digoxigenin (Roche Applied Science) and detected with rhodamine-conjugated anti-digoxigenin to locate the inserted transgenes. The slides were mounted in Vectashield (Vector Laboratories) containing DAPI2 counterstain. The cells were examined and analyzed using a Zeiss epifluorescence microscope with appropriate filters, and images were captured using Cytovision imaging equipment and software (Applied Imaging Corp., Santa Clara, CA). Metaphase and interphase nuclei were analyzed for localized signals and chromosome identification achieved by analysis of the reversed DAPI banding pattern.

RT-PCR Analysis—Total RNA was prepared using the Tri-Reagent BD system (Molecular Research Center, Cincinnati, OH) as described by the manufacturer. cDNA was synthesized using random hexamers as primers. Primers used to amplify the sequence between exon 1 and exon 2 were: forward primer 5’-CGGCTGAGGAAAGCTCTCGCTTCATGACGATC-3’ and reverse primer 5’-CCACCATGATGGTCTCCAAAGCTA-3’.

The PCR was performed using the following conditions: denaturation at 94 °C for 30 min, annealing at 55 °C for 30 s, and extension at 72 °C for 2 min. After 30 cycles, the PCR products were resolved on a 2% Nusieve GTG-agarose gel. The above primers amplified a 170-bp and a 189-bp products from the huβ-globin gene, which correspond to the normal and aberrantly spliced mRNA, respectively.

Sequencing of RT-PCR Products—The PCR-amplified products were digested with XhoI and EcoRI and cloned into the respective sites of pBluescript-KS+. Colonies were picked based on blue/white color selection and verified by sequencing using Big Dye Terminator Kit V3.1 (PerkinElmer) according to manufacturer’s specifications and using the RT-PCR forward primer as above. In brief, sequencing PCR was performed using the following conditions: rapid thermal ramp to 96 °C, hold at 96 °C for 30 s, rapid thermal ramp to 50 °C, hold at 50 °C for 15 s, rapid thermal ramp to 60 °C, hold at 60 °C for 4 min. After 25 cycles, rapid thermal ramp to 4 °C.

Hematological Analysis—Blood was collected from transgenic mice ≥8 weeks of age by retro-orbital bleeding. Full blood examination was performed on whole blood collected into tubes containing EDTA. Full blood examination was performed using an automated Roche Applied Science Cobas Helios hematological analyzer at the Walter and Eliza Hall Institute, Melbourne, Australia. A minimum of 2 weeks was allowed between repeat samples, when such samples were necessary, to minimize the distortion of hematological parameters by frequent bleeding. Cellulose acetate gel electrophoresis was used to visualize hemoglobin tetramers. Samples containing approximately equal amounts of hemoglobin were run on cellulose acetate membranes (Helena Laboratories). Hemoglobin bands were visualized using Ponceau S stain.

2 The abbreviations used are: DAPI, 4’,6-diamidino-2-phenylindole; RT-PCR, reverse transcription PCR; HPLC, high-pressure liquid chromatography; SR, serine/arginine-rich; AO, antiseisme oligonucleotide; BM, bone marrow.
Measurement of Globin Chain Synthesis—The measurement of \( \text{h}^\beta \)-globin and \( \text{h}^\beta \)-globin chain synthesis was performed by high-pressure liquid chromatography (HPLC) as described previously (25). Red blood cells (20 \( \mu \)l) were lysed in 500 \( \mu \)l of lysis buffer (0.1 \( \mu \)M 2-mercaptoethanol, 0.1 \( \mu \)M HCl) and then made 1 ml with 500 \( \mu \)l of 50% aqueous acetonitrile. The lysate was centrifuged, and the supernatant was analyzed by HPLC. Globin chain separation was performed by loading 30 nM nitrile. The lysate was centrifuged, and the supernatant was analyzed by tandem mass spectrometry to confirm globin chain identity. Peak protein fractions were collected and analyzed by tandem mass spectrometry to confirm globin chain type.

RESULTS

Creation and Characterization of BAC Transgenic Mice—In this study, our aim was to establish a humanized mouse model carrying the IVSI-110 splicing mutation and determine whether the \( \text{h}^\beta \)-globin pre-mRNA produced in mice was aberrantly spliced as in humans. Founder mice carrying either the normal or IVSI-110 genomic fragment were identified by screening genomic DNA by \( \text{h}^\beta \)-globin-specific PCR (data not shown) and mated with C57BL/6 mice to generate F1 germ-line transgene progeny. The transgenic mice were bred with C57BL/6 mice to generate F1 germ-line transgene progeny. The transgenic mice were genotyped by multiplex PCR using genomic DNA. Three genes were amplified by PCR (Fig. 2A). The bottom band represents the \( \text{h}^\beta \)-globin gene (198 bp), the middle band represents the HPRT knock-out cassette (315 bp), and the top band corresponds to the \( \text{h}^\beta \)-globin gene (447 bp). Transgenic mice on a \( \text{h}^\beta \)-globin background were obtained for both transgenic lines, whereas viable transgenic mice on a \( \text{h}^\beta \)-globin background could only be generated with the normal \( \text{h}^\beta \)-globin locus. We have previously reported that homozygosity for two copies of the normal locus was necessary to rescue the embryonic lethal \( \text{h}^\beta \)-globin locus. We were unable to identify any IVSI-110 transgenic on a \( \text{h}^\beta \)-globin background at birth indicating that the level of globin expression is insufficient to support fetal growth to birth. This is because of the low level of \( \text{h}^\beta \)-globin expression caused by the IVSI-110 mutation.

Genotype Determination—To determine the degree of hematological complementation of the \( \text{h}^\beta \)-globin locus in a thalassemic environment, transgenic mice were bred onto a \( \text{m}^\beta \text{th} \cdot 3/3^+ \) and a \( \text{m}^\beta \text{th} \cdot 3/3^+ \) background. The transgenic mice were genotyped by multiple PCR using genomic DNA. Three genes were amplified by PCR (Fig. 2B). The bottom band represents the \( \text{h}^\beta \)-globin gene (198 bp), the middle band represents the HPRT knock-out cassette (315 bp), and the top band corresponds to the \( \text{h}^\beta \)-globin gene (447 bp). Transgenic mice on a \( \text{h}^\beta \)-globin background were obtained for both transgenic lines, whereas viable transgenic mice on a \( \text{h}^\beta \)-globin background could only be generated with the normal \( \text{h}^\beta \)-globin locus. We have previously reported that homozygosity for two copies of the normal locus was necessary to rescue the embryonic lethal \( \text{m}^\beta \text{th} \cdot 3/3^+ \) mouse, with the mice displaying normal hematological parameters as measured by full blood examination. We were unable to identify any IVSI-110 transgenic on a \( \text{m}^\beta \text{th} \cdot 3/3^+ \) background at birth indicating that the level of globin expression is insufficient to support fetal growth to birth. This is because of the low level of \( \text{h}^\beta \)-globin expression caused by the IVSI-110 mutation.

Transgene Copy Number Determination—Two transgenic lines were analyzed by quantitative radioactive PCR. Because the human and the mouse globin genes are very similar at the DNA sequence, the primers were designed to amplify both the \( \text{h}^\beta \)-globin and \( \text{m}^\beta \)-globin genes. However, only the amplified mouse PCR product contains an internal Ncol restriction site, which was used to differentiate between the mouse- and human-specific \( \beta \)-globin PCR product. The PCR products generated using the HM primers set were digested with Ncol and separated by agarose gel electrophoresis (Fig. 2C). The two lower bands represent the two mouse-specific products, 125 and 242 bp, whereas the top band represents the undigested \( \text{h}^\beta \)-globin gene, 381 bp. As expected, the wild type Ncol-digested mouse control PCR produced

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**Fluorescence in Situ Hybridization Analysis of Transgenic Mice**—Transgenic mice carrying the normal or the IVSI-110 \( \text{h}^\beta \)-globin transgene were analyzed by fluorescence in situ hybridization. Metaphase spreads of fibroblast cells were prepared from F0 progeny. The transgenic founders were all found to contain a single integration site (Fig. 2A). The integrated BAC DNA was located using labeled insert DNA, and chromosome identification was made on the basis of the banding pattern obtained from the inverted DAPI metaphase image. In the founder containing the normal \( \text{h}^\beta \)-globin locus, the transgene was proximally located on chromosome 14, whereas in the founder containing the IVSI-110 locus the transgene was medially located on chromosome 1 (Fig. 2A).

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**FIGURE 1.** The map of the \( \text{h}^\beta \)-globin locus contained in a 183-kb genomic fragment. A, the 183-kb fragment contains seven olfactory receptor (OR) genes upstream of the locus control region and the hypersensitive site 5′-HS-111, located \( \sim 111 \) kb upstream of the e-promoter. Cfr9I restriction sites are indicated by C and the SfiI restriction sites are indicated by S. A 36 kb fragment is released after Cfr9I digestion, containing the \( \gamma^\text{c}, \gamma^\text{s}, \delta, \) and \( \beta \)-globin genes. B, Southern blot mapping of transgenic mice containing the normal \( \text{h}^\beta \)-globin locus (left panel) and the IVSI-110 \( \text{h}^\beta \)-globin locus transgenic mice. C, schematic diagram indicating transgene copy number and proposed orientation corresponding to the normal (top) and IVSI-110 (bottom) \( \text{h}^\beta \)-globin locus.
two mouse-specific PCR products, whereas the human control PCR produced one human-specific PCR product. The relative intensity of the hu-β-globin-specific PCR product was compared with the mu-β-globin-specific PCR products on a wild type background (which contains four adult β-globin genes) and on a mu-β-thal background (which contains two adult β-globin genes). Using this approach, the transgene copy number was determined. Both transgenic lines contained two copies of the hu-β-globin locus (Fig. 2C).

Expression of hu-β-Globin in Adult Transgenic Mice—The transgenic lines containing the normal or IVS1-110 hu-β-globin locus were bred with mu-β-thal mice to generate mice hemizygous for the mu-β-globin locus on a mu-β-thal background. The expression level of the mu-β-globin chain was 10% in wild type transgenic mice and 35% in hemizygous transgenic mice on a heterozygous knock-out background (Fig. 3B). Importantly, further breeding produced viable mu-β-thal mice expressing only the hu-β-globin locus (mu-β-thal/hu-β) (Fig. 3A), thus confirming that the 183-kb genomic fragment, containing the hu-β-globin locus, can fully complement mu-β-thal mice. Measurement of globin chain synthesis in the humanized IVS1-110 mouse on wild type background was below measurable levels as seen by HPLC (data not shown); however, hu-β-globin chain synthesis by the humanized IVS1-110 mouse on mu-β-thal background expressed hu-β-globin at ~3% relative to α-globin chain. It was noted that the level of hu-β-globin chain synthesis in the humanized IVS1-110 transgenic mouse was ~90% lower compared with hu-β-globin chain synthesis in the humanized normal hu-β-globin transgenic mouse on a similar background. These results suggest that this mouse model accurately recapitulates the β-globin gene defect that is typical for IVS1-110 β-thalassemia patients.

In addition, there was a significant increase in the level of hu-β-globin chain in the mu-β-thal mice compared with normal wild type mice as detected by HPLC globin chain separation and cellulose acetate gel electrophoresis (Fig. 3). This is thought to be because of post-translational events, where reduced synthesis of mu-β-globin chain in the mu-β-thal mice results in reduced competitive binding between the hu-β-globin chain for α-globin resulting in an increase in the level of chimeric hemoglobin.

Detection of Aberrant Splicing—Splicing of hu-β-globin pre-mRNA in the humanized IVS1-110 mouse model was examined. RT-PCR was performed on RNA derived from whole blood, BM, and spleen from the humanized IVS1-110 mouse model and compared with RNA derived from blood isolated from a homozygous IVS1-110 β-thalassemia patient (Fig. 4). PCR primers specific for the hu-β-globin gene were used to amplify the correctly spliced hu-β-globin mRNA spanning the 3’-end of exon 1 to the 5’-end of exon 2 regions. As a control we used a transgenic mouse containing the normal μ-β-globin (Fig. 4).

As determined by RT-PCR transgenic mice that containing the IVS1-110 β-globin locus displayed one aberrantly spliced product. A detailed sequence analysis of cloned BM RT-PCR products revealed that the +110 mutation was recognized as the expected 3’-splice site. This aberrant splice product was identified in BM, spleen, and blood of mice as well as in the blood of IVS1-110 β-thalassemia patients. We noted quantitative differences in the amount of aberrant RT-PCR splice products amplified by PCR. In the BM and spleen, the +110 splice product was present in greater amounts compared with correctly spliced β-globin mRNA, whereas in the blood of IVS1-110 mice and IVS1-110 β-thalassemia patients the normal splice product was the predominant mRNA species. Presumably the difference in relative mRNA levels is because of mRNA instability.
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We compared the strength of the authentic and aberrant splice sites using the Shapiro and Senapathy (S&S) matrix (26). The +110 3ss scored 80.1, whereas the natural 3ss scored 84.5 (Fig. 4). Although the +110 3ss scored lower than the natural 3ss, it is not clear how the spliceosome can selectively discriminate against the authentic 3ss in preference for the weaker aberrant splice site. There are several studies, which report that RNA sequence elements bound by serine/arginine-rich (SR) family of proteins promotes the use of 5’- and 3’-splice sites through protein-protein and protein-RNA interactions. SR proteins can bind to the pre-mRNA. Intronic binding of SR proteins has previously been reported to inhibit splicing (27). Because the IVSI-110 mutation promoted intron splice factor; SR protein SF2/ASF, SC35, SRp40, and SRp55 (28). Although other SR protein-binding sites have been reported, the ESEfinder predicted that the IVSI-110 mutation disrupted an SRp40 binding motif. At this stage, we can only speculate that the IVSI-110 mutation may have disrupted the activity of an SR protein repressor.

Full Blood Examination—A full blood examination was performed on transgenic mice carrying the normal or IVSI-110 huβ-globin locus, including red blood cell count, total hemoglobin, hematocrit, erythrocyte indices, and reticulocyte counts. A summary of the results is shown in Table 1. The *mu*βth-3/+ mice were found to be anemic, as reflected in the marked decrease in red blood cells, hematocrit, and hemoglobin concentration. The reticulocyte counts in *mu*βth-3/+ mice were significantly higher when compared with wild type mice, indicating active erythropoiesis. When the *mu*βth-3/+ mice carry the normal huβ-globin locus, the levels of red blood cells, hematocrit, and hemoglobin concentration increase to levels similar to wild type mice. We also noted that reticulocyte counts of *mu*βth-3/+ mice carrying the huβ-globin locus decreased approaching wild type levels (Table 1). The low levels of huβ-globin chain synthesis by the humanized IVSI-110 transgenic mouse failed to complement the hematological abnormalities in full. However, the small amount of huβ-globin chain synthesis (~3%) by the humanized IVSI-110 mouse generated a small but measurable improvement in hematological values, which were statistically significant (Table 1).

**DISCUSSION**

Transgenic mice containing large genomic fragments are increasingly being developed as in vivo model systems to facilitate our understanding of the biology and etiology of human diseases. With most of the human genome available as BAC clones, humanized BAC/YAC transgenic mice are increasingly the in vivo model systems of choice to map regulatory regions and to unravel the molecular mechanisms underlying developmental regulation and expression of functional genomic loci (2–6). With the growing demand for more sophisticated murine models to emulate human disease, BAC transgenics in combination with other gene knock-out technologies are seen as important experimental tools for disease modeling.

In this study, we demonstrated that mice transgenic for the human β-globin locus, with the IVSI-110 G → A mutation produce the same aberrant spliced product found in IVSI-110 β-thalassemia patients. We noted qualitative differences in the level of aberrant +110 RT-PCR splice product between bone marrow and peripheral blood. Although the bone marrow sample showed more aberrant +110 RT-PCR splice product relative to normal β-globin, the blood samples derived from IVSI-110 transgenic mice and IVSI-110 β-thalassemia patients exhibited the reverse. We attribute the difference in relative ratios of RT-PCR products to mRNA instability. The +110 aberrant splice product generates a premature stop codon, which is known to trigger the nonsense-mediated mRNA decay process. This pathway degrades mRNAs containing premature termination codons generated by point mutations and/or splicing alterations, whose translation product may be detrimental to the cell (29).

Although large genomic transgenes are likely to contain all of the necessary regulatory elements required for tissue-specific and position-
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TABLE 1
Full blood examination

| Groups           | Hb   | RBC  | MCV | MCH | RDW | HCT | RET |
|------------------|------|------|-----|-----|-----|-----|-----|
| Wild type (C57BL/6) (n = 10) | 15.0 ± 1.0 | 9.4 ± 2.0 | 48.7 ± 1.2 | 15.3 ± 0.6 | 13.5 ± 1.1 | 48.5 ± 2.7 | 2.6 ± 1.5 |
| mu^β^-/ mu^β^- (n = 10) | 7.8 ± 0.2 | 5.4 ± 0.4 | 41.7 ± 3.0 | 14.6 ± 0.8 | 34.1 ± 1.0 | 22.2 ± 1.3 | 8.7 ± 3.9 |
| IVS1-110^−/ IVS1-110^− (n = 8) | 15.5 ± 0.9 | 9.7 ± 0.2 | 53.3 ± 1.4 | 16.3 ± 1.1 | 16.1 ± 1.1 | 50.9 ± 0.9 | 3.6 ± 1.1 |
| IVS1-110^−/ IVS1-110^− (n = 8) | 8.6 ± 0.6 | 5.5 ± 0.6 | 44.2 ± 1.3 | 15.6 ± 0.8 | 32.7 ± 0.8 | 24.4 ± 2.3 | 9.3 ± 3.0 |

Student’s t test
p = 0.001 NS p = 0.04 p = 0.04 p = 0.002 p = 0.02 NS

independent expression, mouse models using such transgenes do not always mirror the expression of the endogenous gene in humans (25, 30). The exact reason for this is not clear, but it may reflect the level of transcriptional compatibility between the two species. We next investigated mu^β^-/ carrying two copies of the IVS1-110 β-globin locus (mu^β^-/ + IVS1-110+R/0). Human β-globin chain synthesis was found to be ~3% relative to total β-globin chain synthesis. The low level of huβ-globin chain synthesis by the mu^β^-/ + IVS1-110+R/0 mouse failed to complement the hematological abnormalities. However, the small amount of huβ-globin chain synthesis produced a small but measurable improvement in hematological values. When we compared the level of huβ-globin chain synthesis between the normal and IVS1-110 mu^β^-/ mice, the IVS1-110 mutation produced a 90% reduction in human β-globin synthesis. Interestingly, this 90% reduction of huβ-globin synthesis in mice is equivalent to the reduction of IVS1-110 β-globin chain observed in IVS1-110 β-thalassemia patients.

Despite the many mutations causing alternative splicing identified to date, the reasons why mutations vary from mild to severe in their effects are poorly understood. Splicing relies on the correct recognition of cis-elements (the 5′- and 3′-splice sites and the branch point), but these are not sufficient to define exon-intron boundaries. Additional cis-acting elements such as exonic or intronic splicing enhancers and silencers significantly influence normal and alternative splice site selection. These elements are recognized by trans-acting factors, including the SR proteins and human ribonucleoproteins (hnRNPs) (27). We can only speculate that the IVS1-110 mutation may have disrupted the binding of an intronic silencer, reducing exon definition and allowing the recruitment of the splicing machinery to upstream aberrant 3′-splice sites.

Kole and colleagues (32) were the first to attempt therapeutic modulation of alternative splicing by targeting antisense oligonucleotides (AO) to the aberrant splice site to restore splicing specificity. This strategy prevents the selection of the aberrant splice site, thus favoring the recognition of the correct splice site by the splicing machinery. By using in vitro and cellular assays, they demonstrated inhibition of aberrant splicing in two common β-thalassemia mutations, IVS1-654 and IVSII-705 (31, 32). In the case of the IVS1-110 mutation, efficient inhibition of aberrant splicing was achieved not by targeting the aberrant splice site, but the natural branch point sequence of the first intron (32). More recently, AO have been shown to correct aberrant splicing in vivo using a transgenic mouse model in which enhanced GFP expression was interrupted by an intron containing IVSII-654 splicing mutation (33). A systemic delivery of AO restored enhanced GFP expression in all tissues that absorbed AO, providing evidence that AO can be used in vivo to restore normal splicing. They found correction of aberrant splicing was variable for different tissues, but this may reflect the levels of transgene expression. Although this animal model represents an excellent in vivo model system for the investigation of delivery and activity of various AO chemistries, it is not clinically relevant with respect to anemia and extramedullary erythropoiesis.

An alternative approach to restoring splicing specificity, lost through mutations, would be the use of bifunctional AOs. By combining an antisense-targeting domain and peptide-recruiting domain it may be possible to recruit specific splicing factors that might influence splicing in a positive or negative manner. This approach has recently been shown to specifically restore wild type splicing when directed to defective BRACA1 or SMN2 pre-mRNA (34, 35).

In this study, we showed that this humanized IVS1-110 mouse model recapitulates the splicing defect that is typical for β-thalassemia patients carrying the IVS1-110 mutation. We showed that the humanized transgenic mouse mu^β^-/ carrying the IVS1-110 huβ-globin locus, represents a physiologically relevant in vivo model system, which can provide a unique opportunity for the development and evaluation of various therapeutic strategies such as AO and pharmacological-based therapies.

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