Inactivation of Mouse α-Globin Gene by Homologous Recombination: Mouse Model of Hemoglobin H Disease


We have disrupted the 5' locus of the duplicated adult α-globin genes by gene targeting in the mouse embryonic stem cells and created mice with α-thalassemia syndromes. The heterozygous knockout mice (-α/-αα) are asymptomatic like the silent carriers in humans whereas the homozygous knockout mice (-α/-α) show hemolytic anemia. Mice with three dysfunctional α-globin genes generated by breeding the 5' α-globin knockouts (-αα/α) and the deletion type α-thalassemia mice (-/-αα) produce severe hemoglobin H disease and they die in utero. These results indicate that the 5' α-globin gene is the predominant locus in mice, and suggest that it is even more dominant than its human homologue.

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The human α-globin locus is located on chromosome 16 and is arranged in the order of ζζ/ζζζζαααα. The common molecular mechanism giving rise to α-thalassemia is caused by deletion of the α-globin structural genes. Because the α-globin genes are duplicated, α-thalassemia could result in three phenotypes. When one of the four α-globin genes in the diploid genome is deleted (−α/−αα), a clinically silent carrier state results. When two of the α-globin structural genes are deleted either in cis (−αα/−ααα) or in trans (−α/−αα), the phenotype of α-thalassemia trait ensues. Deletion of three of the α-globin genes (−αα/−ααα) causes hemoglobin H (HbH) disease, which manifests as a mild to moderate hemolytic anemia, and deletion of all four α-globin genes (−αα/−αααα) results in hydrops fetalis, which is usually incompatible with life. α-Thalassemia can also be caused by point mutations. Most point mutations that inactivate the α-globin gene affect the α-globin gene by homologous recombination in mouse embryo-derived stem (ES) cells and generated mice with the genotype of (−αα/−ααα). Studies of the phenotypes of these mice indicate that the 5' α-globin gene in the mice is also dominant and even more dominant than its human homologue.

MATERIALS AND METHODS

Construction of the targeting plasmid. The mouse adult α-globin genes were isolated from the λ- phage library of the strain 129sv mouse (Strategene, La Jolla, CA). Various phage clones spanning the entire α1- and α2-globin genes were isolated and the DNA purified. To prepare the gene-targeting construct, the plasmid pPNT, which contains a neomycin resistance gene (neo) and a herpes simplex virus thymidine kinase gene (HSVtk), was used as the backbone. The genomic fragments of 4.2 kb from the EcoRI to the BamH1 sites at the 5' side and the 4.5 kb from the BamHI to the EcoRI sites of the 3' side of exon 2 of the α1-globin gene are linked to the 3' and 5' end of neo gene, respectively. The resulting plasmid, pPNT-α1, with HSVtk, served as a positive selectable marker, was linearized at the Not I site, and was used in the gene-targeting experiment (Fig 1).

ES cell line and electroporation. Gene targeting by homologous recombination was performed with JM1 ES cells isolated from the inbred strain 129sv mice (The Jackson Laboratory, Bar Harbor, ME). The cells were cultured on embryonic fibroblasts as feeder layers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal bovine serum, 0.1 mmol/L of 2-mercaptoethanol, 2 mmol/L of glutamine, and 0.1 mmol/L of nonessential amino acids. Purified leukemia inhibitory factor (GIBCO, Gaithersburg, MD) was added to the culture medium at 103 U/mL. Cells, 105, were electroporated with the targeting plasmid at 20 mg/L in the complete ES cell culture medium in a cuvette of 5-mm long, 100-mm2 cross section chamber in the gene pulser electroporater (Bio-Rad, Richmond, CA). A single pulse of 240 V, 25 μF was used. Transfected cells were cultured overnight in a nonselective medium and then subjected to selection with G418 at 200 mg/L (GIBCO) and ganciclovir at 2 μmol/L (Syn Tex Corp, Palo Alto, CA). Single colonies were picked at around 10 to 12 days and cells expanded in 24-well dishes. Aliquots of each clone cells were frozen as stocks and the rest expanded for DNA analysis.

Screening for homologous recombination. Southern blot analysis was used to detect homologous recombination. Genomic DNA was extracted from ES cell clones, digested with the restriction enzymes HindIII or BglII (New England Biolabs, Beverly, MA), and separated on a 0.8% agarose gel. DNA fragments were depurinated with acid and transferred in the alkaline medium onto a Hybond N+ nylon membrane according to the manufacturer's recommendation (Amersham, Arlington Heights, IL). The filter was hybridized with a BamHI fragment of 1.3 kb, which is located 5.5 kb 5' to mouse α-globin.
MOUSE α-GLOBIN GENE KNOCKOUTS

Fig 1. Targeted disruption in the mouse α1-globin gene locus by homologous recombination. (A) Wild-type mouse α-globin genomic arrangement on the top line, pNT-α1 is the targeting construct containing the HSVtk and neomycin resistance genes, and the arrow points to the targeted genomic structure and the lengths of the wild-type (wt) and recombinant (rec) restriction fragment using the probe marked p H. HindIII; Bg; BglII; B BamHI; E, EcoRI. (B) Southern blot analysis of the wild-type ES cells and three different targeted clones (3, 33, and 38).

5'α-globin gene as probe and the homologous recombination events identified.

Hemoglobin analysis. For adult mice, blood was collected by retroorbital puncture. Hematologic measurements such as the hematocrit (Hct), hemoglobin concentration (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and the reticulocyte count (Retic %) were done (Steritech, Concord, CA). For day 12.5 to 18.5 embryos, blood was obtained by cutting the jugular vein, flushing the blood with phosphate-buffered saline and spinning down the blood cells. Hemoglobin was analyzed by isoelectric focusing electrophoresis using the kit (Isolab Inc, Akron, OH) with modification. Whole blood was lysed and the red blood cell lysate separated on an agarose gel with a pH gradient of 6 to 8. The gel was run on a mini-gel apparatus (Bio-Rad) at 800 V for 30 minutes, and the voltage then increased to 1,200 V for an additional 10 minutes. The proteins were fixed and hemoglobins visualized unstained. The intensity of various bands was scanned and measured by Image Quant (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Gene targeting in the mouse α1 (5' α)-globin locus. The targeting scheme is shown in Fig 1. The DNA construct, pNT-α1 containing both neo and HSVtk genes, was used to target the 5' α-globin gene in the ES cells by positive/negative selection scheme. Twenty micrograms of the targeting plasmid was linearized by Not I and introduced to 1 × 10⁶ cells by electroporation. Of the 120 G418-resistant colonies, 35 were resistant to both G418 and ganciclovir and 3 colonies (3, 33, and 38) were shown to be homologous recombinants by Southern blot analysis. HindIII digestion gives an endogenous band of 13 kb and correctly targeted band of 14.3 kb. Similarly, the BglII digest gives an endogenous band of 10 kb and a targeted band of 7 kb. Targeting efficiency of 8.6% and approximately fourfold enrichment by using TK gene was obtained in this experiment.

Phenotype of α1-globin gene knockout mice. All three recombinant cell lines were injected into blastocysts of C57BL/6J mice and chimera mice obtained from each of the three lines. Subsequent mating of the chimeric and C57BL/6J mice showed the transmission of correctly targeted α1 locus in the offspring. When two of the heterozygous α1 knockout mice were mated, three genotypes (aa/aa, α/α, and α/α) were seen. The hematologic indices were measured and they are shown in Table 1. A mild thalassemia phenotype is apparent in the heterozygous knockout mice as indicated by the borderline decrease in MCV, similar to that sometimes observed in human silent carriers. The thalassemia phenotype is more severe in the homozygotes as indicated by decreased Hb, lower MCV and MCH, and increased reticulocyte count . When compared with the α-thalassemia mice, Hbα⁺⁻/⁻ (genotype of --/αα) from The Jackson Laboratory, the homozygous α1 knockout mice were more hemolytic as shown by the higher reticulocyte count. The Hbα⁺⁻ mice were generated from chemically induced mutation that resulted in deletion of the entire embryonic and adult α-globin genes and the flanking regions. Homozygosity for this mutation is lethal at early embryonic stage.

Table 1. Hematologic Data of α1 Globin Gene Knockout Mice

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Genotype</th>
<th>Hb (g/dL)</th>
<th>MCV (fL)</th>
<th>MCH (pg)</th>
<th>Reticulocytes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>αα/αα</td>
<td>14.65 ± 1.34</td>
<td>56.71 ± 0.99</td>
<td>16.93 ± 0.43</td>
<td>2.58 ± 0.84</td>
</tr>
<tr>
<td>Heterozygote</td>
<td>*α/αα</td>
<td>13.74 ± 0.80</td>
<td>50.97 ± 1.88</td>
<td>15.11 ± 0.55</td>
<td>2.33 ± 1.50</td>
</tr>
<tr>
<td>Homozygote</td>
<td>*α/αα</td>
<td>13.20 ± 0.69</td>
<td>45.05 ± 1.22</td>
<td>12.15 ± 0.50</td>
<td>11.33 ± 0.58</td>
</tr>
<tr>
<td>Hbα⁺⁻/⁻</td>
<td>--/αα</td>
<td>12.75 ± 1.82</td>
<td>46.10 ± 2.51</td>
<td>13.45 ± 1.21</td>
<td>5.02 ± 0.82</td>
</tr>
</tbody>
</table>

All values are expressed as mean ± SD. In each group all hematologic indices were calculated based on five mice except the reticulocyte value of the homozygous group, in which only three mice were used.
Hemoglobin analysis of $\alpha_1$-globin gene knockout mice. Hemoglobin of normal, heterozygous, and homozygous $\alpha_1$ knockout mice was analyzed by isoelectric focusing electrophoresis (Fig 2A). An extra band of more negatively charged species was detected only in the homozygous $\alpha_1$ knockout mice, amounting to approximately 15% of the total hemoglobin. The band was eluted and analyzed on a Triton acid urea gel and shown to consist of only the $\beta$-chain (data not shown). This $\beta_4$ hemoglobin was not seen in the Hb$\alpha^h,-3$ mice, indicating that the $\alpha_1$ locus is more dominant. When heterozygous $\alpha_1$ knockout mice were mated with the Hb$\alpha^h,-3$ mice, the doubly heterozygous genotype was not seen in 20 liveborns, although this genotype is expected to be found in 25% of the offspring. This suggests that the genotype may be embryonic lethal. We then studied the 12.5- to 19.5-day embryos of such matings. Of the 57 fetuses examined, 16 had the doubly heterozygous genotype. This result shows that the genotype $(\alpha/\alpha^-)$ is not compatible with life (Table 2).

Hemoglobin analysis of mice from HbH mating. We analyzed the hemoglobins of the 17.5-day embryos with four genotypes obtained from the mating of $\alpha/\alpha^h$ with $\alpha^-/\alpha^h$. Not present in other three genotypes, hemoglobin H in mice homozygous for the $\alpha^h$ locus may even be polychromasia, and targeting, characteristic of severe thalassemia (Fig 4A and B). Brilliant cresyl blue stain showed very high reticulocyte counts as well as HbH inclusions in virtually all of the red blood cells (Fig 4C and D).

Table 2. Mating of *$\alpha/\alpha$ and $\alpha^-/\alpha$ Mice

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Genotype</th>
<th>Liveborn</th>
<th>Embryo (12.5 d-19.5 d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>$\alpha^h/\alpha^h$</td>
<td>6</td>
<td>13</td>
</tr>
<tr>
<td>Heterozygote</td>
<td>$\alpha^h/\alpha^-20$</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Heterozygote</td>
<td>$\alpha^-/\alpha^h$</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>Double heterozygote</td>
<td>$\alpha^-/\alpha^h$</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
<td>Total no.</td>
<td></td>
<td>20</td>
<td>57</td>
</tr>
</tbody>
</table>

Data were obtained from two litters for the liveborn and seven litters for the embryo.

DISCUSSION

In these studies we sought to produce mouse models for the human $\alpha$-thalassemia syndromes. By using homologous recombination in embryonic stem cells, we have disrupted the $5'$ $\alpha$-globin gene of the mouse. Analysis of the phenotype of mice produced by matings of these $\alpha_1$ knockout heterozygotes and comparison with the complete $\alpha$-globin deletion mice ($\alpha^-/\alpha^-)$ from The Jackson Laboratory showed the following: The single $\alpha$-globin knockout ($\alpha^-/\alpha^h$) mice are not hemolytic, as indicated by the normal reticulocyte counts, although they had borderline anemia and borderline MCV. This genotype, therefore, mimics the silent carrier state of human $\alpha$-thalassemia. However, homozygosity for the $\alpha_1$ knockout ($\alpha/\alpha^-20$) begins to show hemolysis, as shown by the elevated reticulocyte count. There is some anemia but a notable decrease in MCV and MCH. HbH could be readily shown by electrophoresis. When these homozygous mice were compared with the deletion type $\alpha$-thalassemia mice, Hb$\alpha^h,-3$ mice, no difference in hemoglobin levels was observed, presumably because of efficient compensation of the hemolytic process by the two $\alpha$-globin genes in each genotype. However, the latter type showed less severe hemolysis. Although some HbH inclusion bodies were shown by electron microscopic studies in the Hb$\alpha^h,-3$ mice, hemoglobin H $(\beta_4)$ was not detected by isoelectric focusing electrophoresis. This confirms that, as in humans, the $5'$ $\alpha$ gene is the more dominant locus in mice.

Additional studies on mice derived from mating between the $\alpha_1$-globin knockouts and the $\alpha$-thalassemia, Hb$\alpha^h,-3$ mice show that the $5'$ a locus in mice may even be more dominant than its human homologue. This is already indicated by the hemolytic anemia and the demonstration of HbH in mice homozygous for the $\alpha_1$ knockout ($\alpha^-/\alpha^-20$). In contrast, homozygosity of the silent carrier state ($\alpha/\alpha^h$) in humans is not accompanied by hemolytic anemia or HbH in the adult. Furthermore, as shown in Table 2,
Fig 3. Embryos at 19.5 days of gestation obtained from a mating of heterozygous 5'-α-globin gene knockout and an Hba<sup>−/−</sup> mouse. The smaller and paler embryos on the bottom row were shown to have the α/− genotype.

Fig 4. Blood smears from day 17.5 embryos with normal (A and C) or HbH (B and D) genotype. (A) and (B) are stained with the Wright stain and (C) and (D) with brilliant cresyl blue. Original magnification × 1,000.
mice with a genotype of \( \alpha/-\) have severe hemolytic anemia, HbH inclusions in virtually all of their red blood cells, and do not survive as newborns. The equivalent condition is HbH disease caused by the compound heterozygous state between a deletion and a 5' \( \alpha \) structural gene mutation such as HbH(\( \alpha^+ \)) or an initiation codon mutation.\(^{13}\) These patients do have HbH disease, but they only suffer from mild to moderately severe anemia. In fact, they survive to adulthood with only a fraction of their red blood cells showing HbH inclusions.\(^{14}\)

Why does the 5' \( \alpha \)-globin gene knockout in mice produce such a severe phenotype? It is now believed that the high level of globin expression in erythroid cells is caused by the interaction between the individual structural gene and the locus control region (LCR), which is located upstream 5' to the globin structural genes.\(^{15-19}\) Also, the genes closer to the LCR may interact more readily with the LCR and, therefore, be expressed at higher level.\(^{20,21}\) This is an explanation for the dominance of the the 5' \( \alpha \)-globin gene. In addition, mutations that deleted the 5' gene may be less severe than mutations that inactivate the upstream gene by point mutations or insertions because the LCR will interact more freely with the 3' gene in the former case, whereas the LCR may continue to interact with the nonfunctional 5' \( \alpha \)-gene and decrease its chances of interacting with the 3' gene in the latter case.\(^{22}\) In support of this explanation, HbH(\( \alpha^+ \)) is more severe than HbH because of three-gene deletion.\(^{23,24}\) Also, initiation codon mutation of the 5' \( \alpha \)-globin gene in the human is more severe than initiation codon mutation of the 3' \( \alpha \)-globin gene.\(^{25}\) However, HbH disease in the mouse produced by targeted disruption (\( \alpha^+/-\)) is even more severe, as these mice die in utero with HbH inclusions in all the red blood cells. This finding, together with the fact that \( \alpha \)/*\( \alpha \) mice have hemolytic HbH disease, suggests that the 5' \( \alpha \)-globin gene in the mouse is more dominant than in the human. An explanation may lie in the distance between the two \( \alpha \)-globin gene loci. Whereas the human \( \alpha \)-globin loci are separated by approximately 4 kb, the distance between the two mouse \( \alpha \)-globin genes are 12 kb. The mouse \( \alpha \)LCR, as the human \( \alpha \)LCR is located 5' to the \( \alpha \)-globin loci.\(^{26}\) Hence, the 5' \( \alpha \)-globin gene would have an even greater chance of interacting with the \( \alpha \)-globin LCR and, therefore, be the more dominant locus when compared with its human homolog. Alternatively, it is conceivable that the deletion outside the \( \alpha \)-globin locus may contribute to the severity of this HbH phenotype. Recently, Paszty et al\(^{27}\) have generated \( \alpha \)-thalassemia mice with deletion of both \( \alpha I \) and \( \alpha 2 \) globin genes by gene knockout technology. It would be interesting to compare the phenotype of the HbH mice generated from this gene with our present model. It is known that the human \( \alpha \)-globin loci are located in CpG-rich islands whereas no such islands found in the mouse \( \alpha \)-globin genes.\(^{28}\) The erythroid-specific transcription factor, GATA-1 binding protein, was identified in the promoter of the 5' \( \alpha \)-gene in the mouse but not in the human \( \alpha \)-globin gene. Hence, the dominance of the 5' \( \alpha \)-globin gene in mice and humans could also be due to differences in chromosomal environment or DNA-protein interactions.

REFERENCES

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