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24. Supported in part by NIH grants AM-34357, AM-17702, and 5-R01-GM19328. S.M.L. is recipient of a research career award (5 K06-GM14208) and J.R.C. of a research career development award (AM 1016) of the National Institutes of Health. We thank S. Nagase and M. Inoue for helping to establish the analbuminemic rat colony, R. Chervu for assistance in carrying out the radionuclide scanning studies, and D. Mitra for technical assistance.

13 December 1985; accepted 15 May 1986

Correction of Murine β-Thalassemia by Gene Transfer into the Germ Line

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A murine β-thalassemia was corrected by the transfer of cloned β-globin genes into the mouse germ line. The cloned mouse β^{maj}-globin gene or the cloned human β-globin gene was introduced into mice deficient in β-globin synthesis because of a deletion of the β^{maj}-globin gene. Both introduced genes produced functional β-globin chains, leading to a reduction in one case, and elimination in another case, of the anemia and associated abnormalities of the red blood cells.

HUMAN β-THALASSEMIA IS A HEreditary disease in which insufficient β-globin chains are produced in red blood cells, because of a variety of lesions in the β-globin gene. Although the severity of the resulting anemia varies according to the level of β-globin chains produced, many forms of β-thalassemia lead to childhood fatality. No satisfactory treatment has yet been devised (*1*). A murine model of human β-thalassemia, which has recently been described, may provide a valuable experimental system for the development of new treatments for human thalassemia. The mutation responsible for murine β-thalassemia arose spontaneously in a mouse of the β-globin haplotype Hbb^{d}, which includes the two adult β-globin genes β^{maj} and β^{min}. In the mutant haplotype, $Hbb^{th\text{-}1}$, the β^{maj} gene is entirely deleted but the β^{min} gene remains intact. Animals homozygous for this deletion produce a reduced amount of β-globin, and consequently suffer from a hemolytic anemia similar to a human β-thalassemia of intermediate severity (*2*).

It has been suggested that β-thalassemia might eventually be treated by the insertion of the normal β-globin gene into the hemopoietic stem cells and its consequent expression in red blood cells of affected individuals (*3*). While cloned genes have been successfully introduced into murine hemopoietic cells by means of retroviral vectors (*4*), the efficient expression of a globin gene introduced in this manner has not yet been achieved. In contrast, when introduced into the mouse germ line via the fertilized egg, cloned β-globin genes have been expressed specifically in erythroid cells, often at very high levels (*5, 6*). Although the correction of genetic deficiencies by transfer of genes into the germ line (*7*) is not a feasible strategy for human gene therapy (*3*), we thought it useful to attempt to correct the murine β-thalassemia by this procedure, if only as a preliminary step toward eventual somatic gene therapy for human thalassemia.

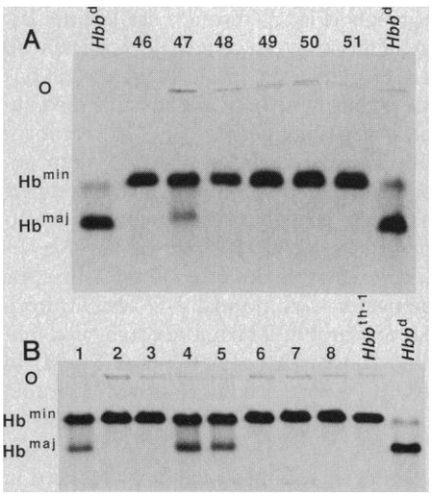

Fig. 1. Expression of the cloned β^{maj}-globin gene in transgenic mice. (A) Cellulose acetate electrophoresis of cystamine-modified hemolysates (*8*) from two normal mice (Hbb^{d}) and six mice (MB46–MB51) developed from homozygous $Hbb^{th\text{-}1}$ zygotes that were microinjected with β^{maj} gene. Mice MB47 and MB51 are transgenic. (B) A similar analysis of eight offspring (1–8) of mouse MB47 mated to a homozygous $Hbb^{th\text{-}1}$ female mouse.

Two different experiments were performed, one utilizing the cloned mouse β^{maj}-globin gene and the other the cloned human β-globin gene. In the first experiment, a 7.0-kb Eco RI fragment containing the mouse β^{maj}-globin gene (*8*) was microinjected into homozygous $Hbb^{th\text{-}1}$ mouse zygotes. A total of 129 eggs were transferred to foster mothers, ten mice were born, and two (mice MB47 and MB51) carried multiple copies of the microinjected gene, as determined by Southern blot analysis. When hemolysates of peripheral blood were analyzed by cellulose acetate electrophoresis (*9*), one of the two transgenic mice (MB47) was found to synthesize a significant amount of β^{maj}-type hemoglobin (Fig. 1A). This animal, a male, was mated to homozygous $Hbb^{th\text{-}1}$ females, and several of the progeny were found to synthesize β^{maj} hemoglobin, indicating that they inherited and expressed the microinjected gene (Fig. 1B). The mouse β^{maj} gene continued to be transmitted as a Mendelian trait and expressed at a similar level in subsequent generations.

While the ratio of β^{maj} to β^{min} hemoglobin is 4:1 in normal mice homozygous for the Hbb^{d} haplotype, and in $Hbb^{d}/Hbb^{th\text{-}1}$ heterozygotes it is 1:1 (*2*), in these transgenic animals the ratio was approximately 1:2. Thus, the introduced β^{maj}-globin genes in this transgenic line produced fewer globin chains than a single endogenous β^{maj} gene.

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Nevertheless, the expression of the introduced genes appears to have caused a significant reduction of the anemia. While the homozygous Hbb^{th-1} mice displayed a reduced hematocrit and hemoglobin level, as well as an elevated reticulocyte count, the transgenic animals were closer to normal by these criteria (Table 1).

In a second experiment, we introduced the human β-globin gene (a 7.7-kb Hind III fragment) (*10*) into the thalassemic mice to ask whether it could substitute for the missing β^{maj}-globin gene. As we had previously produced several transgenic mice carrying this human gene, and two of these mice synthesized large amounts of human β-globin messenger RNA in erythroid cells (*6*), we were able to introduce the human β-globin gene into the homozygous Hbb^{th-1} background by breeding. A male mouse from transgenic line HB56, homozygous for the Hbb^{s} β-globin haplotype (*11*) and carrying 50 to 100 copies of the human β-globin gene unlinked to the *Hbb* locus, was mated to a homozygous Hbb^{th-1} female.

Several F1 offspring that inherited the human gene were again crossed to homozygous Hbb^{th-1} animals, and hemolysates from the F2 progeny were screened by cellulose acetate electrophoresis. The F2 progeny were either homozygous Hbb^{th-1} as indicated by the absence of the "single" type hemoglobin, or heterozygous Hbb^{s}/Hbb^{th-1} (Fig. 2). In addition, some of the mice contained a novel hemoglobin (Hb X) that migrated faster than any of the normal mouse hemoglobins. This novel hemoglobin has been observed only in transgenic lines known to produce high levels of human β-globin mRNA (*6*). In homozygous Hbb^{th-1} animals, Hb X accounts for approximately 50% of total hemoglobin.

Table 1. Hematologic parameters of blood from normal, β-thalassemic, and transgenic mice. Mean values are shown ± SEM's. The number of animals analyzed is shown in parentheses. Blood was collected from the tail, the hematocrit (Hct) was determined by the microhematocrit method, and hemoglobin (Hgb) and reticulocyte count (Retics) were determined as described (*14*). Str., strain.

β-globin locus	Transgene	Hct (%)	Hgb (g/dl)	Retics (%)
Hbb^{s}/Hbb^{s} (Str. C57BL/6J)	None	40.0 ± 2.1 (8)	12.3 ± 0.5 (8)	4.1 ± 1.0 (8)
Hbb^{th-1}/Hbb^{th-1}	None	33.6 ± 2.0 (8)	10.3 ± 0.4 (8)	32.1 ± 7 (12)
Hbb^{s}/Hbb^{th-1}	None	40.7 ± 0.3 (3)	12.8 ± 0.4 (3)	3.4 ± 1.4 (3)
Hbb^{th-1}/Hbb^{th-1}	β^{maj} (line MB47)	39.5 ± 1.9 (5)	11.6 ± 0.3 (3)	15.3 ± 1.6 (3)
Hbb^{th-1}/Hbb^{th-1}	Human β (line HB56)	45.0 ± 2.4 (5)	14.1 ± 0.6 (5)	10.4 ± 3.9 (5)

To determine its globin chain composition, we eluted the Hb X band from a cellulose acetate plate and analyzed it by electrophoresis on a urea-acid-Triton polyacrylamide gel (*12*). Hb X consisted of human β-globin and mouse α-globin chains, in equal proportion (Fig. 3). Thus, human β-globin chains are able to associate with mouse α-globin chains to form a hybrid hemoglobin tetramer.

Hematological analysis of homozygous Hbb^{th-1} mice carrying the human β-globin gene showed that the thalassemic phenotype was corrected (Table 1 and Fig. 4). The hematocrit and hemoglobin were elevated from thalassemic levels to levels somewhat higher than those of control Hbb^{s}/Hbb^{s} mice, and the reticulocyte count was reduced to a value only slightly above normal. In addition, the severe morphological abnormalities (anisocytosis and poikilocytosis) characteristic of thalassemic murine red

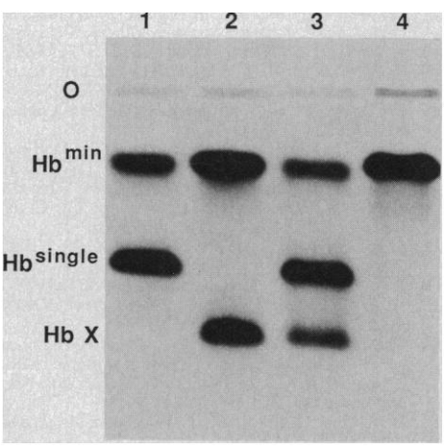

Fig. 2. Detection of a novel hemoglobin in transgenic mice carrying the human β-globin gene. Cystamine-modified hemoglobins were separated by electrophoresis on cellulose acetate (*8*). Lanes 1 to 4, the four types of hemoglobin patterns observed in the F2 generation when a transgenic mouse (HB56) carrying the human β-globin gene on a homozygous Hbb^{s} background, was twice backcrossed against homozygous Hbb^{th-1} mice. The mice analyzed in lanes 2 and 3 have inherited the human β-globin gene, the animals in lanes 2 and 4 are homozygous for the Hbb^{th-1} deficiency, and the animals in lanes 1 and 3 are heterozygous; O, origin.

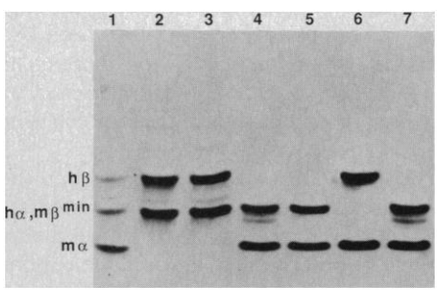

Fig. 3. Unfractionated hemolysates or hemoglobin bands eluted from cellulose acetate strips after electrophoretic separations (see Fig. 2) were analyzed by electrophoresis on a 12% polyacrylamide gel containing 2% Triton X-100, 8*M* urea, and 5% acetic acid (*12*). The gel was then stained with Coomassie blue. Lane 1, total hemolysate from a mouse homozygous for the Hbb^{th-1} mutation and also containing the human β-globin gene (the same sample analyzed in Fig. 2, lane 2); lanes 2 and 3, total human hemolysate containing hemoglobin A; lanes 4 and 5, total hemolysate from a homozygous Hbb^{th-1} mouse. Human α- and mouse β^{min}-globin chains comigrate on this gel system. The two hemoglobin bands from a mouse of the genotype shown in Fig. 2, lane 2, were eluted from cellulose acetate into water, lyophilized, and applied to this gel. Lane 6 contains the lower band, Hb X, which is shown to consist of human β- and mouse α-globin chains. Lane 7 contains the upper band, which consists of mouse β^{min}-globin and α-globin chains; h, human; m, mouse.

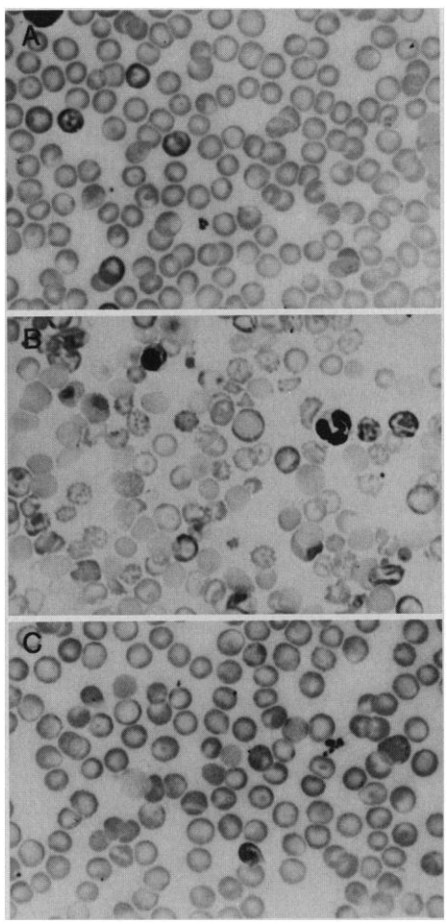

Fig. 4. Blood films from (A) a normal mouse, (B) a homozygous Hbb^{th-1} mouse, and (C) a homozygous Hbb^{th-1} mouse carrying the human β-globin gene, stained with Wright's stain.

Fig. 5. Newborn mice inheriting (left) or failing to inherit (right) the human β-globin transgene. Both mice are homozygous for the Hbb^{th-1} deficiency at the mouse β-globin locus.

blood cells (Fig. 4B) were entirely eliminated by the introduction of the human β-globin gene (Fig. 4C). While neonatal Hbb^{th-1} homozygotes can be recognized by their extreme pallor, transgenic siblings inheriting the human β-globin gene could easily be distinguished by their normal pink color (Fig. 5).

We suspected that the mild polycythemia (elevated hematocrit) in the transgenic mice, compared to control siblings, might be related to the oxygen affinity of the hybrid human/mouse hemoglobin. In man, polycythemia is sometimes caused by mutant hemoglobins with abnormally high oxygen affinities, which release oxygen less readily to the tissues and thus lead to the overproduction of red cells (*13*). Indeed, hemoglobin from the transgenic mice (50% Hb X and 50% Hb^{min}) was found to have a lower P_{50} (or higher oxygen affinity) than any of the normal mouse hemoglobins (Table 2).

These studies demonstrate that cloned adult β-globin genes introduced into murine erythroid cells through the germ line are capable, in some animals, of producing enough functional β-globin chains to overcome a genetic deficiency in β-globin synthesis. The thalassemia was essentially eliminated in the transgenic line (HB56) carrying the human β-globin gene, and partially corrected in the transgenic line (MB47) carrying the cloned mouse β^{maj}-globin gene. This difference can be attributed primarily to the different levels of expression of the introduced genes in these two particular transgenic lines. The levels of expression of cloned β-globin genes in different transgenic mice have been observed to vary widely, apparently as a function of the chromosomal locus at which the genes are integrated (*5, 6*). Although there does not appear to be a strong correlation between the level of expression and the number of copies of an exogenous β-globin gene in transgenic mice (*5, 6*), both of the transgenic lines described in this report carried 50 or more gene copies, and correction of β-thalassemia by transfer of a single copy of the β-globin gene has not yet been achieved.

It is clear that transfer of genes into the germ line does not represent an appropriate strategy for the correction of human thalassemia or other genetic diseases, for reasons that have been discussed (*3, 7*). Even the approach of gene transfer into hemopoietic stem cells faces severe difficulties, particularly for a disease such as thalassemia where a specific α/β-globin chain balance must be restored. Cloned β-globin genes transferred into the erythroid cells of a thalassemic patient by any route are likely to be expressed at variable levels from cell to cell, a problem that may not be overcome until there is a better understanding of globin gene regulatory mechanisms. While our studies do not eliminate these difficulties, they demonstrate the potential of a cloned β-globin gene to substitute for a defective gene in thalassemic red blood cells, and the lack of an inherent limitation on the efficient and functional expression of transferred β-globin genes.

A more immediate consequence of this work may stem from our ability to replace a large fraction of endogenous mouse hemoglobin with hemoglobin containing a foreign β chain. We are currently producing transgenic mice carrying the mutant human β^S-globin gene responsible for sickle cell anemia (*13*). If a human β^S/mouse α hybrid hemoglobin polymerizes at low oxygen tension, as does human Hb S, it may be possible to convert the β-thalassemic mouse into a murine model of sickle cell anemia.

Table 2. Oxygen affinities of normal and transgenic mouse hemoglobins.

β-globin haplotype	Transgene	Hb (%)	P_{50}*
Hbb^s/Hbb^s	None	100 Hb^{single}	42 (40, 44)
Hbb^d/Hbb^d	None	80 Hb^{maj}	
		20 Hb^{min}	34
Hbb^{th-1}/Hbb^{th-1}	None	100 Hb^{min}	36.2 (35.5, 37)
Hbb^{th-1}/Hbb^{th-1}	Human	50 Hb^{min}	
	β-globin	50 Hb X	27.5 (25.5, 29.5)

*P_{50} is the partial pressure of oxygen at which hemoglobin is 50% oxygenated, and is inversely related to oxygen affinity. The value shown is the mean of two individual measurements (in parentheses), performed on a Hemox analyzer (TCS Medical Products).

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15. We thank A. Bank for many helpful discussions, L. van der Ploeg for comments on the manuscript, R. Popp for the Hbb^{th-1} mutant mice, and S. Ford and W. Tilton for assistance with hematological measurements. Supported by grants from the NIH (HD17704) and the National Foundation—March of Dimes (MDBDF 5-410), an Irma T. Hirschl Career Scientist Award (to F.C.), and fellowships from NATO and from the Cooley's Anemia Foundation (to K.C.).

9 April 1986; accepted 18 June 1986