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Mice deficient in cystathionine β-synthase: Animal models for mild and severe homocyst(e)inemia

(homologous recombination/gene targeting/inborn errors of metabolism/homocysteine)

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ABSTRACT **Studies by various investigators have indicated that elevated levels of plasma homocyst(e)ine are strongly associated with the occurrence of occlusive vascular diseases. With the eventual aim of determining whether or not elevated plasma homocyst(e)ine concentrations are directly causative of cardiovascular diseases, we have generated mice that are moderately and severely homocyst(e)inemic. Homologous recombination in mouse embryonic stem cells was used to inactivate the cystathionine β-synthase [L-serine hydrolyase (adding homocysteine), EC 4.2.1.22] gene. Homozygous mutants completely lacking cystathionine β-synthase were born at the expected frequency from matings of heterozygotes, but they suffered from severe growth retardation and a majority of them died within 5 weeks after birth. Histological examination showed that the hepatocytes of homozygotes were enlarged, multinucleated, and filled with microvesicular lipid droplets. Plasma homocyst(e)ine levels of the homozygotes were ≈40 times normal. These mice, therefore, represent a model for severe homocyst(e)inemia resulting from the complete lack of cystathionine β-synthase. Heterozygous mutants have ≈50% reduction in cystathionine β-synthase mRNA and enzyme activity in the liver and have twice normal plasma homocyst(e)ine levels. Thus, the heterozygous mutants are promising for studying the *in vivo* role of elevated levels of homocyst(e)ine in the etiology of cardiovascular diseases.**

Homocysteine is an intermediate amino acid in methionine metabolism and is either converted to cysteine by transsulfuration or methylated to form methionine. A decreased rate of metabolism through either of these pathways can lead to homocyst(e)inemia and homocystinuria.

The most common type of inherited homocystinuria is caused by a deficiency in cystathionine β-synthase [CBS; L-serine hydro-lyase (adding homocysteine), EC 4.2.1.22]. CBS is the rate-limiting enzyme of the transsulfuration pathway that condenses homocysteine and serine into cystathionine. One in 300,000 newborns is homozygous for a deficiency in CBS (1). The major clinical manifestations of homozygotes include mental retardation, ectopia lentis, osteoporosis, skeletal abnormalities, and fatty liver. Premature arteriosclerosis and thromboembolism are characteristics of patients with severe homocystinuria, and thromboembolic complications are the most frequent cause of death in these patients (1, 2).

Elevated levels of plasma homocyst(e)ine§ have attracted much attention in recent years as a potential risk factor for the development of premature occlusive atherosclerosis in humans. Several studies have found that patients with premature coronary artery disease have significantly higher levels of homocyst(e)ine than control subjects (3–8). Although the role of elevated homocyst(e)ine in the formation of thrombi or in the acceleration of atherogenesis is not well understood, various experiments suggest that homocysteine is a mediator of disease. For example, homocysteine damages cultured human venous and arterial endothelial cells (9, 10). Furthermore, DeGroot *et al.* (11) showed that cells grown from obligate heterozygotes for CBS deficiency were much more sensitive to methionine-mediated injury than were control cells. Other *in vitro* studies have demonstrated that homocysteine enhances autooxidation of low density lipoproteins (12), enhances biosynthesis of thromboxane (13), inhibits cell-surface thrombomodulin expression (14), promotes vascular smooth muscle cell growth (15), and enhances binding of lipoprotein(a) to fibrin (16). *In vivo*, long-term infusion of homocystine in baboons leads to endothelial desquamation, to an increase in platelet consumption, and to arterial lesions (17). However, there are also conflicting data indicating that an increased incidence of vascular diseases was not found in individuals heterozygous for homocystinuria (18, 19).

Determining whether elevated plasma homocyst(e)ine levels are causative of cardiovascular disease or are a consequence of the disease has been difficult because of the lack of an experimental animal model system. We here describe the generation of mice with CBS deficiency by gene targeting in embryonic stem (ES) cells. The resulting homozygous mutant mice having severe homocyst(e)inemia and the heterozygous mutants having moderate homocyst(e)inemia are both useful as models for pathophysiological studies of human diseases, as well as for evaluating the correlation between elevated plasma homocyst(e)ine levels and cardiovascular diseases.

MATERIALS AND METHODS

Cloning of the Mouse *Cbs* Gene and Construction of the Targeting Plasmid. A 142-bp DNA fragment containing exon 3 of the rat *Cbs* gene was amplified from rat genomic DNA with primers designed from published sequences (20). This fragment was used as a probe to screen a λ phage library made from strain 129 mouse genomic DNA. A clone containing a part of the mouse *Cbs* gene was isolated and its restriction map was determined. A partial nucleotide sequence of the clone was determined in order to localize exons of the mouse *Cbs* gene that are homologous to exons 3, 4, and 8 of the rat and human *Cbs* genes (20, 21). In constructing the targeting plasmid (Fig. 1), a 1.4-kb fragment containing exon 2 and a 7-kb fragment containing exons 5–10 were used as the two arms of

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Abbreviations: CBS, cystathionine β-synthase; ES cells, embryonic stem cells.

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§Plasma homocyst(e)ine, or total homocysteine, refers to the sum in plasma of free and bound homocysteine, the homocysteinyl moieties of the disulfides, homocystine and cysteine–homocysteine.

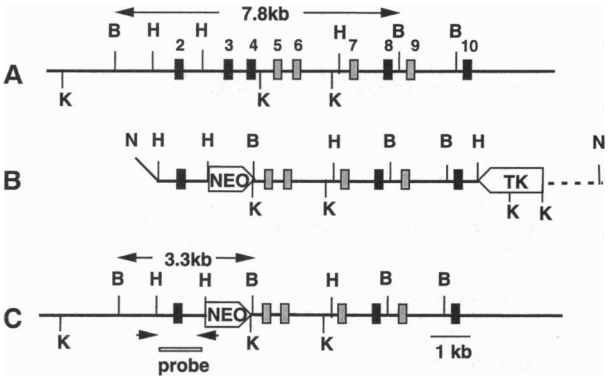

FIG. 1. Targeted disruption of the mouse *Cbs* gene. (*A*) Endogenous *Cbs* gene. Exons 2–10 are shown by boxes and numbered. Exons 2, 3, 4, 8, and 10 (solid boxes) were positioned by hybridization or by nucleotide sequence determination. The other exons (stippled boxes) are diagrammed by analogy to the rat gene (20). (*B*) Targeting construct. *Neo* gene (NEO) replacing exons 3 and 4 is in the same orientation as the *Cbs* gene. Thymidine kinase (TK) gene is oppositely oriented. Broken line indicates vector sequence. A 1.4-kb *Hin*dIII fragment and a 7-kb *Kpn* I/*Hin*dIII fragment were used as the two arms of homology. The *Hin*dIII site of the 7-kb fragment was from the phage vector cloning site. (*C*) Correctly targeted locus depicted after a homologous crossover. Arrowheads indicate positions of the two primers used for PCR analysis. A probe for Southern blot analysis and sizes of *Bam*HI fragments detected by it are indicated. B, *Bam*HI; K, *Kpn* I; H, *Hin*dIII; N, *Not* I.

homology flanking the pMC1neopo1A sequence (Stratagene). The herpes simplex thymidine kinase gene was positioned downstream of the longer arm of homology (22).

Gene Targeting and Production of Mice from Modified ES Cells. BK4 cells, a subclone of E14TG2a (23) derived from strain 129/Ola mice, were cultured on feeder cells as described (24). The construct was linearized with *Not* I and introduced into ES cells by electroporation. Three experiments were carried out, each using 2×10^7 cells and 10 μg of targeting plasmid DNA. Colonies doubly resistant to G418 (200 μg/ml) and ganciclovir (2 μM; a gift from Syntex, Palo Alto, CA) were screened for homologous recombination by PCR (25). Primers 5′-GCCTCTGTCTGCTAACCTA-3′ and 5′-GAGGTCGACGGTATCGATA-3′, corresponding, respectively, to a sequence from the *Cbs* gene outside the targeting construct and to the linker sequence at the 5′ end of the *Neo* gene, gave a 1.5-kb diagnostic PCR product. Southern blot analysis was used to confirm the targeting, using as a probe the 1.4-kb *Hin*dIII fragment shown in Fig. 1. Cells with a disrupted *Cbs* gene were injected into blastocysts to obtain chimeras as described (26). Animals classified as chimeric by coat color were mated with strain C57BL/6J mice, and F_1 animals heterozygous for the disrupted *Cbs* gene were obtained. Mice were handled following the National Institutes of Health guidelines for the care and use of experimental animals and were fed autoclaved chow (RMH 3500; Agway, Syracuse, NY).

Plasma Homocyst(e)ine Measurements. Blood samples obtained from retroorbital bleeding under anesthesia with 2,2,2-tribromoethanol were collected into tubes containing EDTA, aprotinin, and gentamicin as described (27). Plasma was collected by centrifugation and stored at −70°C. Plasma homocyst(e)ine levels were determined as described (4) with 50 μl of plasma.

Student's *t* test of unpaired observations was used to determine statistical significance.

Northern Blotting of mRNA and Enzyme Assay. Livers were isolated from five 21-day-old mice of each genotype. Equal weights of tissues from five animals of each genotype were pooled, and total RNA was extracted according to the method of Chomczynski and Sacchi (28) using RNazol (Tel-Test, Friendswood, TX). Poly(A)$^+$ RNA was prepared as described by Kingston (29). Northern blots were prepared by standard methods (30) and hybridized to a 1.4-kb *Hin*dIII fragment containing exon 2 or a 3-kb fragment containing exons 8 and 9 of the mouse *Cbs* gene or to a PCR fragment containing exon 3 of the rat *Cbs* gene. A cDNA clone for human glyceraldehyde-3-phosphate dehydrogenase (Clontech) was used as a probe to evaluate the amount of mRNA loaded. mRNA levels were estimated by densitometric analysis of autoradiograms after serial exposures.

Equal weights of liver tissues from five animals of each genotype were combined and homogenized in 3 vol of 1.15% KCl. After centrifugation at 10,000 × *g* for 15 min, CBS activity was measured in the supernatant by the method of Kashiwamata and Greenberg (31). Protein concentrations were measured by a dye binding procedure (Bio-Rad).

Histological Analysis. Mice were sacrificed by an overdose of 2,2,2-tribromoethanol, and the heart, eye, lung, liver, kidney, spleen, and knee joint were excised and fixed in 4% paraformaldehyde/0.1 M sodium phosphate buffer (pH 8). The bone was decalcified by immersion in 0.5 M EDTA (pH 8) for 1 week. Paraffin sections of the tissues were stained with hematoxylin and eosin. Frozen sections of the liver were stained with Sudan IVB and hematoxylin.

RESULTS

Targeted Disruption of the Mouse *Cbs* Gene. The targeting plasmid is illustrated in Fig. 1. Swaroop *et al.* (20) have shown that the rat *Cbs* gene produces at least four mRNAs resulting from alternative splicing of transcripts; all the functionally active mRNAs contain exon 3, where Lys-116 resides. The authors showed that the CBS protein shares sequence similarity with other pyridoxal 5′-phosphate-dependent enzymes, including those from lower organisms. Lys-116 is most likely the pyridoxal 5′-phosphate binding residue of CBS because it aligns with the established pyridoxyllysine in other enzymes. We therefore made a targeting construct so that exons 3 and 4 of the mouse *Cbs* gene will be replaced by the bacterial *Neo* gene after the planned targeting (Fig. 1*C*).

A total of 360 colonies doubly resistant to G418 and Ganciclovir were analyzed by PCR, and 13 candidate targeted cell lines were identified. Modification of the *Cbs* locus was confirmed by Southern blot analysis of DNA from these cells after digestion with various restriction enzymes including *Bam*HI, *Kpn* I, and *Cla* I (data not shown). Four cell lines were injected into recipient blastocysts, and 16 chimeras were generated. Three of these chimeras transmitted the modified *Cbs* gene to the next generation.

Mice Deficient for CBS. The resulting heterozygotes grew normally and were outwardly healthy. But when the heterozygotes were bred to produce homozygotes for the disrupted gene, we found that the homozygotes did not thrive. At weaning (postnatal day 21), the ratio of wild-type to heterozygous mice was very close to the expected ratio of 1:2 (80:151). However, the number of homozygotes was significantly lower than expected (42 observed vs. 77 expected; $P < 0.001$). The number of surviving homozygotes decreased to 18 at 4 weeks and to 15 at 8 weeks. Fig. 2 presents the number of homozygotes surviving at weekly intervals after birth as percentage of the expected number. As indicated above, the homozygotes were born at close to the expected percentage, and there was no significant reduction in their number until postnatal day 14. This result suggests that inactivation of the *Cbs* gene does not affect embryonic survival but that it results in a high incidence of death during the 3rd and 4th postnatal weeks.

We followed the growth of pups by measuring their body weights (Table 1). There was no significant difference in body weights among mice of the three genotypes at postnatal days 1 and 7. However, homozygous mutants failed to gain weight

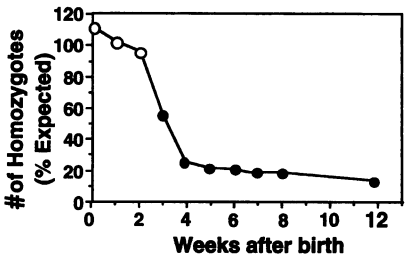

FIG. 2. Number of homozygous mice expressed as a percentage of that expected from numbers of wild-type and heterozygous littermates. On days 1, 7, and 14, pups (n = 55, 42, and 68, respectively) were sacrificed and genotyped by Southern blot analysis (open circles). All remaining animals were genotyped at day 21, and survival of the 42 homozygotes was followed (solid circles).

thereafter, and their body weights at day 14 were ≈80% of the weights of wild-type and heterozygous mice ($P < 0.02$). From day 14 to day 21, homozygotes showed little weight gain, so that there was an even larger difference in body weight between the homozygous mutants and wild-type animals at day 21 ($P < 0.001$). The homozygotes that died at the early postnatal stages were those with lower body weight. They also had characteristics suggestive of growth retardation, such as delayed eye opening and facies typical of very young animals. In addition, their tails and extremities were smaller in diameter relative to their length when compared to wild-type mice.

To date, 12 homozygous mice (4 males and 8 females) have survived >2 months. These mice, in contrast to those that died early, have nearly normal stature at weaning. They maintain a relatively normal body size thereafter until just before death. A strong correlation is, therefore, observed between the severity in growth retardation and the failure to survive in the mice lacking CBS. Two surviving male F_2 homozygotes both reproduced when mated to F_2 heterozygous females. The survival rate of their homozygous offspring was similar to that of the homozygous offspring from matings between the F_1 heterozygotes. None of four homozygous females so far tested has reproduced.

A single transcript was detected by Northern blot analysis of liver mRNA from wild-type and heterozygous animals with a probe made from a PCR fragment containing exon 3 of the rat *Cbs* gene (Fig. 3). The same transcript was also detected by probes containing exon 2 or exons 8 and 9 of the mouse *Cbs* gene. None of these probes, however, showed specific hybridization to mRNA from the homozygotes, indicating that the homozygotes do not make any detectable CBS transcripts. The amount of mRNA in the liver of heterozygotes was estimated to be ≈45% that of wild-type animals by densitometric analysis. Enzyme activity was not detectable in liver homogenates from homozygous mice, while enzyme activity in the liver from heterozygotes was 42% that in the liver of wild-type mice (0.42 vs. 1.00 μmol/hr of cystathionine production per mg of protein of liver extract).

Table 1. Growth of pups from heterozygous F_1 matings

Postpartum age, days	Body weight, g		
	Wild type	Heterozygotes	Homozygotes
1	1.4 ± 0.3 (13)	1.5 ± 0.3 (25)	1.5 ± 0.2 (15)
7	4.3 ± 0.9 (15)	4.0 ± 1.0 (22)	4.2 ± 1.0 (12)
14	7.5 ± 1.2 (20)	7.4 ± 1.9 (31)	6.0 ± 1.3 (17)*
21	9.8 ± 2.3 (29)	9.2 ± 2.4 (56)	6.4 ± 1.3 (16)†

Values are expressed as means ± SD. Mice from 7–14 litters at each postpartum day were weighed, sexed, and genotyped. Numbers of mice are given in parentheses. There were no statistical differences between body weights of males and females.
*$P < 0.02$ against wild-type and heterozygous mice.
†$P < 0.001$ against wild-type and heterozygous mice.

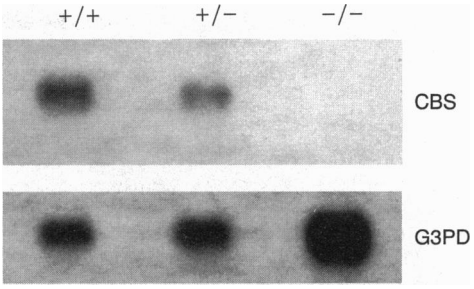

FIG. 3. Northern blot analysis of liver mRNA. Poly(A) RNA (2 μg) isolated from livers of wild-type mice (+/+), homozygous mutants (−/−), and heterozygous mutants (+/−) were electrophoresed, blotted, and hybridized to a DNA fragment containing exon 3 of the rat *Cbs* gene. Amounts of RNA loaded are depicted by hybridization to a cDNA probe for human glyceraldehyde-3-phosphate dehydrogenase (G3PD).

Plasma Homocyst(e)ine Concentrations in CBS-Deficient Mice. The plasma homocyst(e)ine levels in 21-day-old F_2 homozygotes were ≈40 times higher than those of age-matched normal littermates (Fig. 4*A*). Heterozygotes have about twice the normal homocyst(e)ine levels. We also measured the plasma homocyst(e)ine levels in wild-type and heterozygous F_1 male mice at 3, 12, 22, and 44 weeks after birth (Fig. 4*B*). Plasma concentrations decreased with age, reaching levels of 3 nmol/ml and 7 nmol/ml, respectively, by 22 weeks of age; the 1:2 ratio between wild type and heterozygotes was observed at all time points. As the genetic background of all F_1 animals is identical, we conclude that this 1:2 ratio is directly caused by the mutation.

Histological Observations of Homozygous CBS Mutants. Most homozygotes at weaning were runted and their eyes were smaller than normal and not completely open. The 21-day-old homozygous mutants, which were doing well and were vigorous at this time, were sacrificed for histological examination. Gross examination of their organs showed no obvious differ-

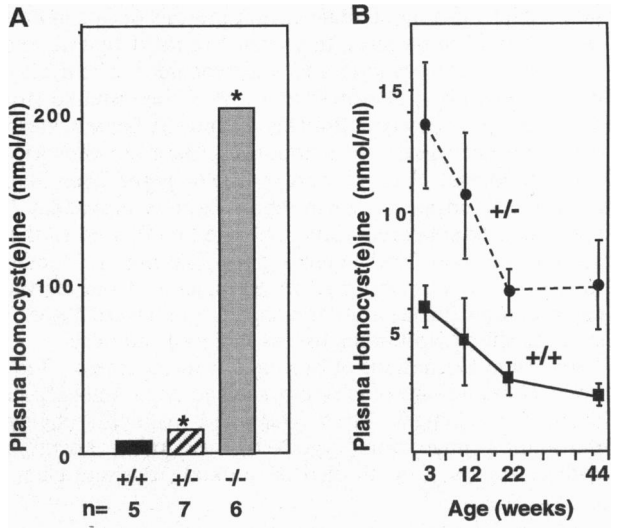

FIG. 4. Plasma homocyst(e)ine levels in CBS-deficient mice. (*A*) Mean plasma homocyst(e)ine levels in 21-day-old F_2 mice. Both females and males are represented. Values were 6.1 ± 0.8 nmol/ml in wild-type (+/+; n = 5), 13.5 ± 3.2 nmol/ml in heterozygous (+/−; n = 7), and 203.6 ± 65.3 nmol/ml in homozygous (−/−; n = 6) mice. *, $P < 0.001$ against wild type. (*B*) Decrease in plasma homocyst(e)ine levels with age. Plasma homocyst(e)ine levels of wild-type and heterozygous F_1 male animals were measured at 12, 22, and 44 weeks of age. Levels at the 3-week time point were obtained from different F_1 males. At all times, levels in heterozygotes are statistically different from those in wild type (n = 5; $P < 0.001$).

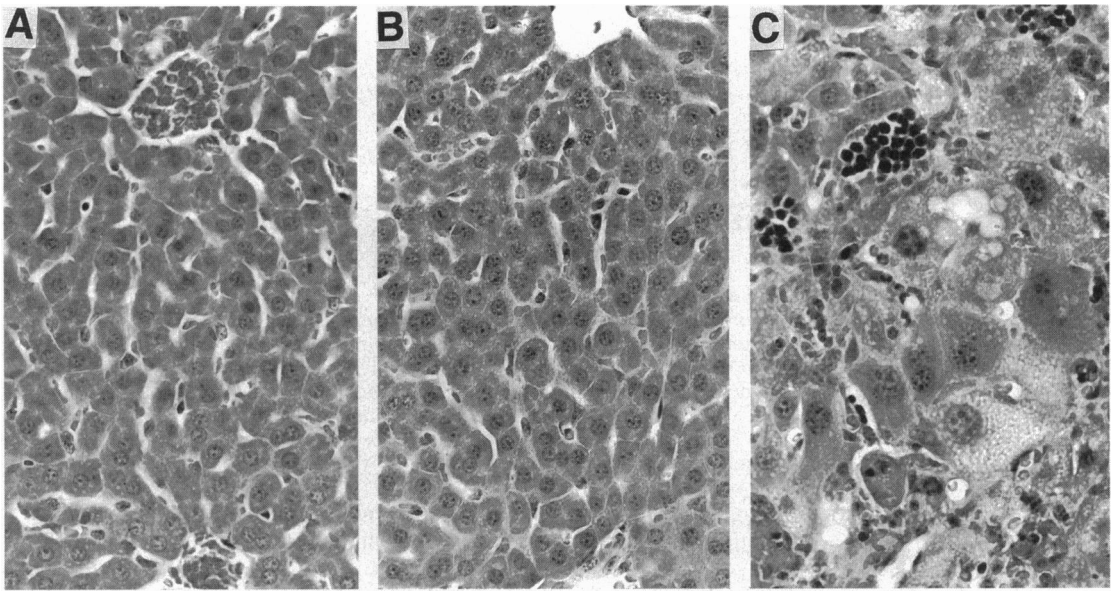

FIG. 5. Hepatic morphology of 21-day-old normal, heterozygous, and homozygous CBS-deficient mice. Normal hepatic morphology consists of uniform hepatocytes with bland nuclei that contain small nucleoli (*A*). In heterozygous mice (*B*), the hepatocytes have anisonucleosis and fairly prominent nucleoli and appear to be slightly larger. Homozygous animals (*C*) have enlarged pleomorphic hepatocytes with large, prominent nucleoli. The hepatocytes are frequently binucleated or multinucleated, and their cytoplasm often contains multivesicular cytoplasmic lipid droplets. (×130.)

ences except that the color of the livers was light tan in contrast to the reddish-brown color of the livers of heterozygotes and wild-type mice.

The morphology of hepatocytes from the 21-day-old homozygous mice was abnormal, as judged by light microscopic observation (Fig. 5). The hepatocytes were enlarged and had an approximately twice normal mean diameter (29.2 ± 11.0 vs. 14.3 ± 2.5 μm). Their nuclei were also enlarged. Multinucleated and binucleated hepatocytes were evident. In some hepatocytes from the homozygous mutants, the cytoplasm was filled with microvesicular lipid droplets. The hepatocytes of the heterozygotes and wild-type mice showed no microvesicular fat droplets. Extramedullary hematopoiesis was prominent in the liver of homozygotes, in contrast to its virtual absence in the livers of heterozygotes and wild-type mice. The livers of 1- and 7-day-old homozygotes were not distinguishable from the livers of their wild-type littermates, but the hepatocytes in 14-day-old homozygotes were abnormal (data not shown).

No pathological changes were notable in the eyes, knee joints, kidneys, lungs, and hearts of homozygous mice at day 21, except that they appeared immature when compared to their 21-day-old wild-type and heterozygous littermates. Hemosiderin deposition was observed in the spleen of one of four 21-day-old homozygous mutant mice. Thrombi were absent in vascular segments and other tissues from all the mice.

Postmortem evaluations of two older homozygotes, a 3-mo-old female and a 6-mo-old female, showed hepatomegaly and mild autolytic changes. Their livers had histologic changes similar to the younger homozygous mice described above. The immediate causes of death of these animals were not clear.

DISCUSSION

Increased levels of plasma homocyst(e)ine are observed as a consequence of several different inborn errors of metabolism, including deficiency in CBS, abnormalities in 5-methyltetrahydrofolate reductase, and several conditions that lead to vitamin B_{12}, vitamin B_6, or folate deficiencies (1). The mutation we have introduced into mice disrupts the *Cbs* gene and completely eliminates expression of the gene, as determined by the absence of enzyme activity and of mRNA in the livers of homozygotes. Plasma homocyst(e)ine levels in the homozygotes were 40 times normal. Heterozygous mutants had half normal levels of enzyme activity in the liver and twice normal levels of plasma homocyst(e)ine.

Levels of plasma homocyst(e)ine and tissue CBS activities are heterogenous among different human patients with CBS deficiencies. The severity of the clinical symptoms also varies significantly. Some patients respond well to the administration of pyridoxine, while others do not (1). At least a part of this heterogeneity is the result of different molecular lesions. For example, individuals homozygous for a G307S replacement are nonresponsive to pyridoxine treatment, while individuals homozygous for an I278T replacement are responsive to pyridoxine (32).

The homozygous mutant mice lacking the CBS enzyme represent the extreme end of the spectrum of human cases. They are born normal but suffer from severe growth retardation; the majority die before 4 weeks of age. The livers of 21-day-old homozygotes are abnormal, characterized by enlarged and multinucleated hepatocytes with lipid droplets and by extramedullary hematopoiesis. Although similar liver hypertrophy and fatty droplet inclusions unaccompanied by fibrosis have been found by liver biopsy or at autopsy in virtually all human patients with homocystinuria (1), they are not the major cause of morbidity. Untreated homozygous mice lacking CBS have more severe liver damage than human patients, and this liver abnormality in mice is likely to contribute to their growth retardation and early death. Growth retardation has been observed in rats fed excessive methionine or homocystine (33), but it is not a characteristic of the human patients. Northern blot analysis of mRNA isolated from the livers of homozygous mutants showed no detectable transcripts for the *Cbs* gene (Fig. 3).

A consistent finding in human CBS deficiency is dislocation of the lens. We noted above that most of the homozygous mutant mice have delayed and narrow eye openings, but no histological abnormalities were obvious in their eyes. Whether other pathological conditions found in human CBS-deficient patients, such as osteoporosis and vascular occlusions, will appear after longer survival of the homozygotes remains to be determined. Restriction of dietary methionine, administration of betaine, or transfer into the mice of a temporarily expressed CBS gene may allow the homozygotes to survive longer. This

in turn would allow the occurrence of the more progressive changes described in humans to be evaluated.

We found that a small fraction of homozygous F_2 animals mature to adulthood. Since these mice are F_2 animals, derived from matings between heterozygous F_1 (C57BL/6J and 129) animals, their genetic compositions differ. It is, therefore, conceivable that there exist other genetic factors that ameliorate the deleterious effect of CBS deficiency. However, against this possibility is our observation that pups resulting from breeding surviving homozygous males to heterozygous females died in roughly the same proportion as in the previous generation. Nongenetic factors, such as birth order, litter size, and body weight at birth may influence the ability to survive.

When considering the elevated plasma levels of homocyst(e)ine as a risk factor for heart diseases, the mean homocyst(e)ine levels in patients with occlusive vascular diseases are 1.3–1.5 times those of controls (8). In this sense, we are encouraged to find that the elevations in heterozygous mice are to levels comparable to those observed in humans. It is, therefore, reasonable to expect that studies of heterozygous CBS-deficient mice will generate important insights into the overall process of atherogenesis. For example, the mutant *Cbs* gene can be introduced into mice lacking apolipoprotein E, which develop atherosclerosis spontaneously (27, 34), to assess the effects of moderate homocyst(e)inemia combined with elevated levels of chylomicron remnants and low density lipoprotein remnants.

In conclusion, we have generated mice carrying the disrupted *Cbs* gene. The homozygous CBS null mice with severe homocyst(e)inemia should be useful for increasing our understanding of the pathophysiology of CBS deficiency, as well as for developing new means of treatment. The apparently healthy heterozygotes with twice the normal plasma homocyst(e)ine levels are promising for studying the *in vivo* role of moderately elevated homocyst(e)ine levels on the etiology of cardiovascular diseases.

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