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ARTICLE *in* HEPATOLOGY · JANUARY 1999

Impact Factor: 11.06 · DOI: 10.1002/hep.510290112 · Source: PubMed

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Mice Deficient in the Urea-Cycle Enzyme, Carbamoyl Phosphate Synthetase I, Die During the Early Neonatal Period From Hyperammonemia

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Ammonia liberated during amino acid catabolism in mammals is highly neurotoxic and is detoxified by the five enzymes of the urea cycle that are expressed within the liver. Inborn errors of each of the urea cycle enzymes occur in humans. Carbamoyl phosphate synthetase I (CPSase I; EC 6.3.4.16) is located within the inner mitochondrial matrix and catalyzes the initial rate-limiting step of the urea cycle. Unless treated, complete deficiency of CPSase I, a rare autosomal recessive disease, causes death in newborn infants. Survivors are often mentally retarded and suffer frequent hyperammonemic crises during intercurrent illness or other catabolic stresses. Biochemically, CPSase I deficiency is characterized by high levels of blood ammonia, glutamine, and alanine, with low or absent citrulline and arginine levels. As a first step toward the development of gene therapy directed to the hepatocyte, we have generated a CPSase I–deficient mouse by gene targeting. Mice with homozygous disruption of CPSase I (CPSase [−/−] mice) die within 36 hours of birth with overwhelming hyperammonemia, and without significant liver pathology. This animal is a good model of human CPSase I deficiency. (Hepatology 1999;29:181-185.)

The hepatic urea cycle detoxifies ammonia liberated by the degradation of amino acids, producing urea.[1] The cycle comprises two mitochondrial enzymes, carbamoyl phosphate synthetase (CPSase I) and ornithine transcarbamylase (OTC), and three cytoplasmic enzymes: argininosuccinic acid synthetase, argininosuccinate lyase, and arginase.[2] The predominant expression of CPSase I and OTC within periportal hepatocytes effectively restricts the urea cycle to the liver.[3] In contrast, the three cytoplasmic enzymes of the urea cycle are expressed in many tissues.

Inherited defects of all of the urea cycle enzymes have been defined in humans, including a knowledge of their individual gene structures, mutations, and biochemical consequences. X-linked OTC deficiency accounts for most cases. The remaining urea cycle enzyme defects are inherited as autosomal recessive conditions. Their combined frequency is estimated to be 1 in 30,000 births, but many cases of fatal neonatal hyperammonemia escape precise diagnosis.[4] Affected babies usually present in the first 24 to 72 hours of life with rapidly fatal hyperammonemia as a consequence of the failure to convert amino acids into urea. A second group of patients with partial gene expression have hyperammonemic crises that occur during adult life during periods of catabolic stress, e.g., surgery, childbirth, and infections.

Ammonia is a potent toxin to neurones and astrocytes within the cerebral cortex and brainstem. Rapid correction of the hyperammonemia is vital if death or brain damage and mental retardation is to be avoided in survivors. Current therapy requires prompt diagnosis, vigorous hemodialysis, ammonia scavengers (sodium phenylbutyrate and benzoate), and intravenous arginine.[5] However, even now, about half of the affected newborn infants with urea-cycle defects die with hyperammonemic coma, and three quarters of survivors have severe mental retardation.[6,7] Survivors require strict dietary protein restriction and lifelong oral therapy with arginine and sodium phenylbutyrate. Orthotopic liver transplantation provides a possible cure, but it has limited availability and significant long-term morbidity. The development of gene therapy for this group of metabolic disorders would have a significant impact, both in the treatment of acute hyperammonemia, and for definitive long-term metabolic correction. Here, we describe the generation of a mouse model of human CPSase I deficiency for the development and testing of gene therapy strategies.

MATERIALS AND METHODS

All animals used in these experiments received humane care under the UK Animals (Scientific Procedures) Act, 1986.

Construction of Targeting Vector (Fig. 1). The production of carbamoyl phosphate from ammonia, carbon dioxide, and water involves two partial reactions, each requiring the binding of adenosine triphosphate. To disrupt the CPSase I gene, a construct was designed to interrupt the sequences encoding the most 5′ nucleotide-binding domain. The mouse CPSase I cDNA and the genomic organization was unknown, although this gene is highly conserved throughout evolution.[8,9] We were able to predict the mouse genomic structure around the most 5′ nucleotide-binding domain by the

Abbreviations: CPSase I, carbamoyl phosphate synthetase I; OTC, ornithine transcarbamylase; PCR, polymerase chain reaction; ES, embryonic stem cell.

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Received May 29, 1998; accepted July 31, 1998.

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Supported by The Wellcome Trust, London, UK, and The Lister Institute of Preventive Medicine, UK (to J.P.S.). Dr. Caskey was an Investigator of The Howard Hughes Institute, Baylor College of Medicine.

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0270-9139/99/2901-0025$3.00/0

homology between the intron positions of the rat CPSase I gene[8,10] and the CPSase III gene from the compressed vertebrate genome Japanese pufferfish, *Fugu rubripes*.[11] (Fig. 2). DNA sequence alignments were used to design oligonucleotide primers to first amplify mouse liver CPSase I cDNA encompassing the most 5′ ATP-binding sequence using polymerase chain reaction (PCR). A 1.5-kb PCR product was sequenced to confirm its identity as encoding CPSase I and used as a probe to isolate genomic clones from a 129/Sv mouse genomic library. Total mouse liver RNA was prepared by the acid-phenol guanidinium thiocyanate method[12] (Trizol, GIBCO-BRL, Rockville, MD), and poly-A^+ messenger RNA purified using oligo-dT cellulose chromatography (Pharmacia, Uppsala, Sweden). The murine CPSase I cDNA around exon 17 was isolated by reverse-transcription PCR of mouse liver messenger RNA, using primers 5′-ATTATGGCCACTGAAGACAGG-3′ (forward) and 5′-AGCACACCAATCAAATTCCAC-3′ (reverse). After cloning the 1.5-kb amplified fragment into a TA cloning vector (InVitrogen, Groningen, Netherlands) and sequencing to confirm its identity, this probe was used to screen a lambda mouse genomic DNA library prepared from 129/Sv mouse DNA (Stratagene, La Jolla, CA). Three overlapping clones were identified and partially sequenced to confirm that they corresponded to the murine CPSase I gene. Based on the physical map and partial sequence data, a targeting construct was designed to insert a PGK*neo*^{r}bpA cassette into a unique *Sfi* I restriction site within exon 17 in the antisense orientation. Two copies of the MC1*tk*bpA cassette were inserted at one end of the homologous sequence to allow for enrichment of targeting events after introduction of the plasmid into embryonic stem (ES) cells.

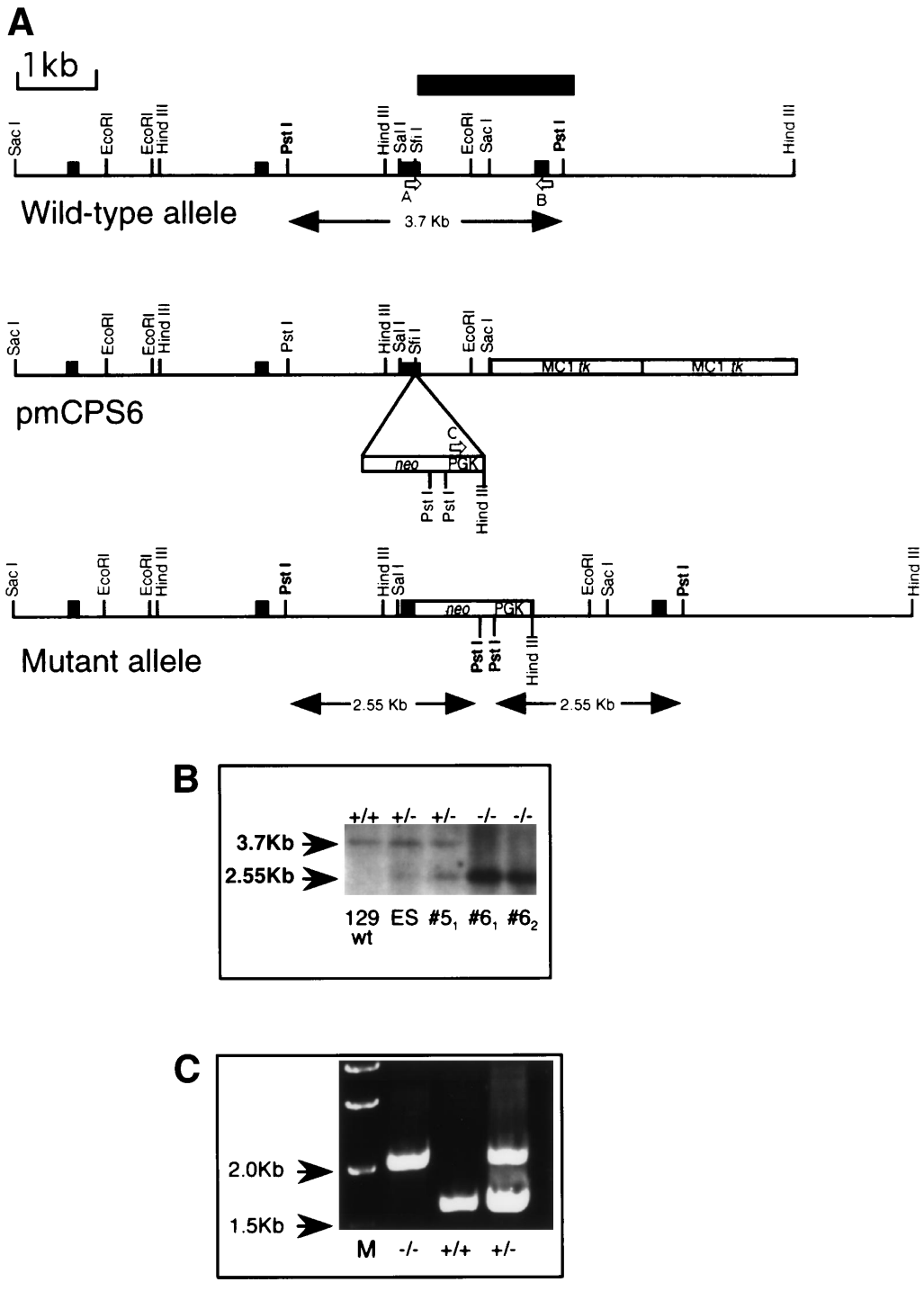

FIG. 1. Disruption of the murine *CPSase I* locus in ES cells and mice. (A) Schematic representation of a segment of the mouse *CPSase I* locus, the CPSase I targeting vector, and the targeted CPSase I locus. Filled boxes indicate predicted exons 15-18, assuming that the mouse *CPSase I* locus is homologous to the rat CPSase I gene. Labeled empty boxes indicate PGK*neo*^{r}bpA and MC1*tk*bpA selection cassettes. The targeting vector was constructed by inserting a neomycin resistance gene (*neo*^{r}) with the phosphoglycerate kinase (PGK) promoter in the antisense orientation into a unique *Sfi* I site of exon 17. Homologous recombination of the targeting vector, which contains 6.2 kb of sequence derived from a 129/Sv mouse CPSase I genomic clone, with the endogenous locus results in the insertion of the 1.6-kb PGK*neo*^{r}bpA cassette. The insertion of two new *Pst* I sites into the targeted locus allows targeted and wild-type alleles to be distinguished by Southern analysis with the indicated probe (black box). Relevant restriction enzyme sites are indicated. PCR primers A, B, and C (see Materials and Methods for DNA sequences) used for rapid genotyping of newborn mice are indicated by *open arrows*. (B) Detection of targeted and endogenous CPSase I alleles by Southern blot analysis of DNA obtained from wild-type (wt) 129/Sv mouse genomic DNA, a correctly targeted ES cell clone, and the offspring from heterozygous intercross matings (#5_1, #6_1, #6_2). DNA was digested with *Pst* I and hybridized with the probe shown in (A). The 3.7-kb *Pst* I fragment corresponding to the wild-type allele is reduced to two identically sized 2.55-kb fragments upon disruption of the locus by integration of the PGK*neo*^{r}bpA cassette. (C) PCR genotyping of newborn mice. Amplification between primers A and B yield a 1.6-kb band in wild-type and heterozygous mice. The insertion of the PGK*neo*^{r}bpA cassette and amplification from a PGK-specific primer, C, with primer B, results in the generation of a larger fragment of 2.1 kb in −/− and +/− animals.

Targeted Disruption of the CPSaseI Gene. The AB1 embryonic stem (ES) cells were cultured, electroporated, and screened by standard methods.[13] Homologous recombination of the linearized targeting vector with the endogenous locus results in the insertion of two new *Pst* I sites into the CPSaseI locus, allowing the targeted and wild-type alleles to be distinguished by Southern analysis with a probe corresponding to intronic DNA just 3′ to exon 17. Targeted ES cell clones were injected into C57BL/6 blastocysts to generate chimeras.[14]

Genotyping of ES Cells and Derived Mice. DNA was prepared from cells by a microplate method,[15] and was analyzed by Southern blotting using standard procedures.[16] DNA obtained from tail biopsies or liver tissue was analyzed by Southern blotting or by PCR using allele-specific primers. The primer set HPTSMEYCII (5′-CACCCCACTTCCATGGAATACTGTATCATT-3′, primer A) (Fig. 1A) with TSACFEPSLD (5′-AATCCAGGCTAGGTTCGAAG-CAGGCTGAGG-3′, primer B) (Fig. 1A) was used to detect the wild-type allele of 1.6 kb; and PGK (5′-ACGTGCGCTTTTGAAGC-GTGCAGAATGCCG-3′, primer C) (Fig. 1A) with TSACFEPSLD to detect the targeted allele of 2.1 kb.

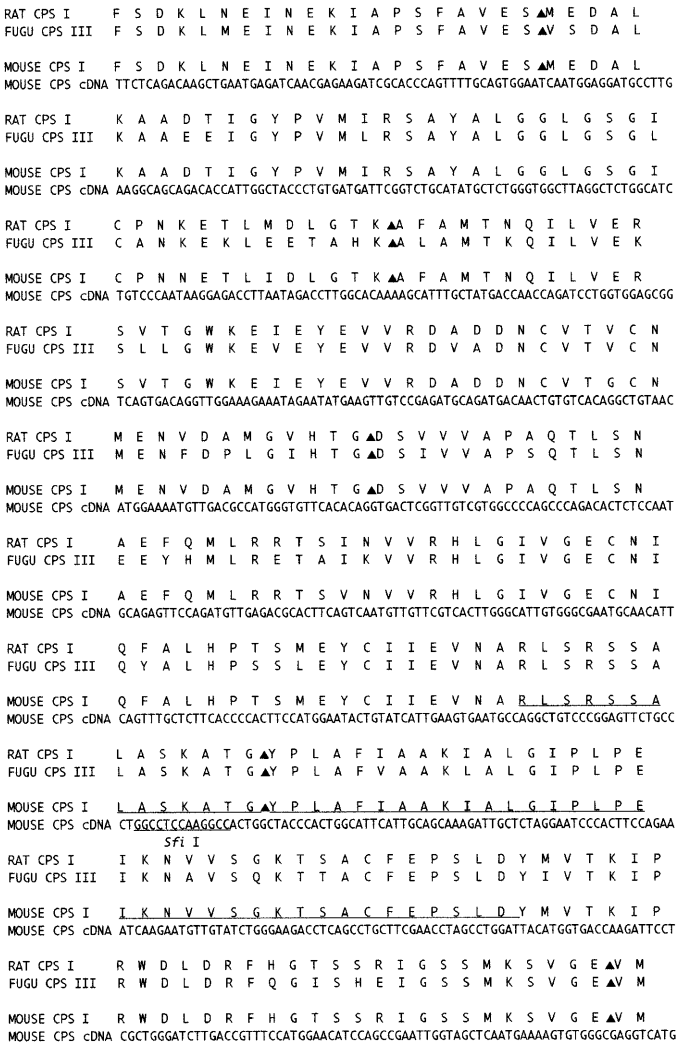

FIG. 2. Sequence alignment of rat CPSase I, *Fugu* CPSase III, and mouse CPSase I. The predicted amino acid sequence is shown together with the cDNA sequence of mouse CPSase I around the proposed 5′ nucleotide binding domain (*underlined*). The conserved intron positions (▲) between the rat and *Fugu* CPS genes correctly predicted the corresponding introns for the mouse CPSase I genomic locus. The amino acid similarity between rat CPSase I and *Fugu* CPSase III facilitated the design of PCR primers to amplify mouse CPSase I from liver cDNA. The PGK*neo*^{r}bpA cassette was inserted into the unique *Sfi* I site to construct the targeting vector (also see Fig. 1).

CPSaseI Enzyme Assay. Mouse liver was flash-frozen in liquid nitrogen and stored at −80°C until analyzed further. Samples were homogenized in 0.1% cetrylammonium bromide plus the protease inhibitors, leupeptin (2 μg/mL) and phenylmethylsulphonyl fluoride (1 mmol/L). CPSase I enzyme activity was measured by coupling to citrulline conversion according to established methods[17] and expressed in micromoles per hour per milligram of protein. Total protein was measured by the bicinchoninic acid assay (Pierce, Chester, Cheshire, UK).

Plasma Ammonia Measurement. Mice were killed within the 6 hours of life, and blood was collected onto a 1-μL drop of 0.5 mol/L ethylenediaminetetraacetic acid on Parafilm to inhibit coagulation. The sample was immediately transferred to an eppendorf tube and briefly centrifuged before carefully removing the plasma layer from the red cells and transferred to a clean tube before flash-freezing in liquid nitrogen. Samples were stored at −80°C. Plasma ammonia was determined by an adaptation of the glutamate dehydrogenase enzymatic method.[18] Briefly, 5 μL of plasma was diluted to 25 μL with sterile water and mixed with 250 μL of assay reagent in cuvette. Following incubation for 3 minutes at room temperature, the optical density at 340 nm was taken, and then 2.5 μL of glutamate dehydrogenase was added to the cuvette, mixed, and incubated again at room temperature for 5 minutes before a second OD_{340} was taken. The decrease in absorbance at 340 nm is directly proportional to the plasma ammonia concentration, and is expressed in micromoles per liter.

RESULTS AND DISCUSSION

The CPSase I gene was disrupted by an insertional targeting vector in AB1 ES cells (Fig. 1A). Of 384 clones that were resistant to both G418 and FIAU, 24 ES cell clones were correctly targeted. Eight independent targeted clones were injected into C57BL/6 blastocysts and four clones generated chimeras that transmitted the mutation in the germline. The chimeras were bred with C57BL/6 mice, the F1 heterozygous mice intercrossed, and their offspring characterized. Mutant mice from all four cell lines had identical phenotypes.

At birth, all littermates appeared healthy and suckled normally, with an easily visible milk-filled stomach through their semitransparent abdominal wall. However, within 12 to 36 hours, one to three mice in each litter were found dead. Southern blot analysis (Fig. 1B) or a more simple PCR assay (Fig. 1C) confirmed that these animals were all homozygous for the null allele (CPSase [−/−]). No CPSase (−/−) pups survived beyond 36 hours.

To characterize the phenotype of the pups, several litters were killed within 12 hours of birth and tissues were collected for biochemical and genetic analysis. Of 86 animals studied, 24 (27%) were CPSase (+/+), 43 (50%) were CPSase (+/−), and 19 (22%) were CPSase (−/−).

The targeted mutation appears to be a null allele, because the mouse liver CPSase I enzyme activity was completely absent from CPSase (−/−) mice. This contention is supported by the absence of a product following reverse-transcriptase PCR using CPSase (−/−) total liver RNA as the substrate (data not shown). Heterozygotes had approximately half of the activity (636 ± 120 μmol/h/mg protein) of their wild-type littermates (1,125 ± 195 μmol/h/mg protein) (Table 1).

The hallmark of CPSase I deficiency is profound hyperammonemia. Because of practical assay limitations, plasma ammonia was undetectable in CPSase (+/+) mice killed within 6 hours of birth. Blood ammonia concentration in

TABLE 1. Plasma Ammonia Concentration and CPSase I Enzyme Activity in Mice Within 6 Hours of Birth

	CPSase I +/+	CPSase I +/−	CPSase −/−
Plasma ammonia (μmol/L)	—	275 ± 67 (7)	4,565 ± 730 (4)
CPSase I enzyme activity (μmol/h/mg protein)	1,125 ± 195 (7)	636 ± 120 (8)	0 (8)

NOTE. The parameters measured and the units in which they are measured are expressed in the first column. The values ± SD are given for each genotype, with the number in parentheses indicating the number of animals analyzed.

heterozygotes was 142 to 365 μmol/L in CPSase (+/−) and 3,616 to 5,630 μmol/L in CPSase (−/−) mice within 6 hours of birth (Table 1). These results are consistent with plasma ammonia measurements from affected patients with complete CPSase I deficiency, reflecting the utility of this animal as a model of the human disease counterpart.

Light microscopy of liver and brain tissue obtained from CPSase (−/−), CPSase (+/−), and CPSase (+/+) mice showed no significant differences, and immunochemistry confirmed an absent signal from CPSase (−/−) mice hepatocytes (data not shown). Electron microscopy of liver samples taken from CPSase (+/+) and CPSase (−/−) littermates killed within 12 hours of birth showed normal mitochondrial morphology (data provided, but not shown). At the time of killing, the CPSase (−/−) mouse had an ammonia level of 4,293 μmol/L, while the ammonia was below the detectable limits for the wild-type control animal. Previous ultrastructural studies on hepatic mitochondria from patients with CPSase I and OTC urea-cycle deficiencies have revealed few pathological features.[19,20] However, one caution that must be raised is that some patients were metabolically stable at the time of liver biopsy.[19] It was proposed that mitochondrial and other subcellular pathology could be more striking during phases of acute or chronic hyperammonemia. One report has described marked ultrastructural differences between the hepatic mitochondria from two female CPSase I–deficient siblings and age-matched controls.[21] Electron microscopy showed that the mitochondria were small and there were more mitochondria per hepatocyte than in control patients, with a concomitant increase in the total mitochondrial volume. Furthermore, the mitochondria of the metabolically stable sibling were of an intermediate size. The authors postulated that because 20% to 30% of hepatic mitochondrial matrix protein is comprised of CPSase I, its deficiency may affect mitochondrial morphogenesis. Our data do not support this observation, although there remains the possibility that this could be a result of species differences between mouse and humans.

CPSase I is unique within the urea cycle: the enzyme functions as a monomeric protein localized to the inner mitochondrial membrane.[2] The second enzyme of the urea cycle, OTC, is active within the mitochondrial compartment as a homotrimer, as is cytoplasmic arginase. The remaining two cytoplasmic enzymes are tetrameric.[2] The *spf*^{ash} mouse is a useful model of partial OTC deficiency, expressing 5% to 15% wild-type levels of active enzyme that fail to aggregate as enzymatically active trimers following mitochondrial import.[22] Morsy et al.[23] reported *in vitro* and *in vivo* data for the correction of the *spf*^{ash} mouse. The *in vitro* admixture of equimolar quantities of native and mutant OTC monomers forms heterotrimers with a 33% reduction in enzyme activity compared with control homotrimers.[24] Although this "spoiler" effect of mutant monomers was overcome by overexpression of the wild-type OTC protein, this creates at least theoretical difficulties for complete correction of other urea-cycle disorders caused by missense mutations affecting multimeric enzymes. When selecting human subjects with urea-cycle disorders for gene therapy, in the first instance, those with very low or absent expression of the multimeric enzyme complex would probably be most amenable to therapeutic correction. In contrast, we would predict that because CPSase I is fully active as a monomer, patients with either partial or complete CPSase I deficiency would be equally amenable to gene therapy. These considerations indicate that the CPSase I–deficient mouse reported here is ideally suited to the development of experimental gene therapy procedures directed toward the liver.

Acknowledgment: This work was initiated at Baylor College of Medicine, and the authors are grateful to the staff within the animal facility for their animal husbandry. They also thank Graham Gatward, Sian Wombwell, and Susan Howard of The University of Cambridge Department of Histopathology for their expert assistance in electron microscopy and histological preparations.

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