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Generation of a Mouse Model for Citrullinemia by Targeted Disruption of the Argininosuccinate Synthetase Gene

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Abstract—*Argininosuccinate synthetase (ASS) is a urea cycle enzyme that forms argininosuccinate from citrulline and aspartate. Mutations at the ASS locus in man cause the inherited disease, citrullinemia. Citrullinemia is inherited as an autosomal recessive trait and is characterized, biochemically, by elevated levels of blood citrulline and ammonia and often results in early neonatal death if untreated. We have used homologous recombination in embryonic stem cells to generate a line of mice having a targeted disruption of the* Ass *gene. Homozygous mutant animals develop high levels of blood citrulline, become hyperammonemic, and die within one or two days after birth. Because the phenotype of the mutant mice closely resembles that of humans who lack the ASS enzyme, we expect that these mice will serve as a useful model for exploring new treatments for citrullinemia including somatic gene therapy.*

INTRODUCTION

Argininosuccinate synthetase (ASS; EC 6.3.4.5) catalyzes the condensation of aspartic acid and citrulline in the presence of ATP to form argininosuccinic acid. This reaction is of primary importance in the urea cycle of ureotelic animals. The urea cycle serves to dispose of the toxic metabolite, ammonia, that results from the breakdown of proteins. The genetic deficiency of ASS in man causes an inborn error of metabolism, citrullinemia, first described in 1962 (1) and reviewed extensively (2, 3). Clinically, citrullinemia is characterized by lethargy, irritability, and poor feeding, proceeding to convulsions, coma, and eventually death if appropriate therapy is not provided. Biochemically, the disease is characterized by hyperammonemia and, as the name implies, massively increased plasma citrulline levels. The disease is heterogeneous both in its time of clinical presentation (ranging in age from day 1 of life to various times within the first decade) and in the molecular basis for the disease (4–6). The disease has been reported in a Friesian line of cattle (7, 8) in Australia.

The therapeutic approach to the management of citrullinemia is based on limiting the amount of protein in the diet and hence the amount of ammonia for disposal and on providing drugs such as phenylbutyrate and benzoate that allow for an alternative pathway for the elimination of excess nitrogen (2). Although this therapy is complex and demanding, it is generally quite effective except when large increases in nitrogen load occur, often due to catabolism associated with infection. In such cases, other, more drastic measures are required, including hemodialysis. In order to provide a model system that would allow for the development

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of more effective therapeutic modalities, including gene therapy, we have developed a mouse model for citrullinemia by employing the procedure of homologous recombination in mouse embryonic stem cells (9).

MATERIALS AND METHODS

Construction of Targeting Plasmid. The targeting vector was derived from a 5.5-kb fragment of the *Ass* gene, containing exons 4 and 5, which was isolated from a BALB/c genomic library (10). A selection cassette containing the neomycin resistance gene under the control of the short form of the RNA polymerase II promoter (11) was inserted in this fragment so as to interrupt exon 4. Two copies of the herpes simplex virus thymidine kinase gene driven by the MC1 promoter (12) were added to the vector to provide for selection against random integration by addition of 0.2 μM 1-[2-deoxy, 2-fluoro-β-*d*-arabinofuranosyl]-5-iodouracil (a gift from Oclassen Pharmaceuticals, San Rafael, California) to the culture medium (13).

Embryonic Stem Cell Culture and Chimera Generation. Three micrograms of the targeting vector were linearized within the vector sequence and was electroporated into AB-1 embryonic stem cells (13). The cells were plated onto mitomycin-C-inactivated STO cells, which express lymphocyte inhibitory factor (14). Following selection, 153 colonies were picked and DNA was prepared as described (15). Successfully targeted cell lines were identified by a polymerase chain reaction assay for a specific junction fragment only generated after homologous recombination. Nine positive colonies were expanded and the homologous recombination event was confirmed by Southern blotting. Cells from two of the colonies were injected into C57BL/6J blastocysts and gave rise to germ line chimeras. Heterozygous offspring from matings of the chimeras with C57BL/6J mice were interbred to produce animals that were homozygous for the disrupted *Ass* gene.

Analysis of Blood and Tissues. Plasma was prepared using ammonia-free heparin, and ammonia concentrations were determined using a glutamate dehydrogenase-based assay kit (Gilford Systems, Oberlin, Ohio). Plasma amino acid levels were determined on a Beckman model 6300 amino acid analyzer. Argininosuccinate synthetase assays were conducted as described previously (16). Livers were removed from animals at autopsy, weighed, and homogenized in five volumes of H_2O. The resulting homogenate was centrifuged and the supernatant fluid removed for assay. The cleared extracts were diluted 1/50 in 0.05 M Tris Cl, pH 7.5, containing 1 mM citrulline prior to assay.

RESULTS AND DISCUSSION

Disruption of Ass *Gene.* We mutated the *Ass* gene in AB-1 embryonic stem cells (13) by means of a replacement-type vector containing a neomycin resistance cassette (Fig. 1A–C). Since we wanted to obtain a null mutant, we placed the neomycin cassette so that it would interrupt one of the first exons in the coding region (exon 4). Two targeted cell lines gave rise to germ line chimeras and transmission of the mutation was confirmed by Southern blot analysis (Fig. 1D). Heterozygotes were interbred and produced the expected 1:2:1 ratio of wild-type, heterozygous, and homozygous offspring (117 animals analyzed).

Functional inactivation of the *Ass* gene was confirmed by assaying the enzyme in the liver (Table 1), and the genotype was monitored by Southern blotting. No enzyme activity was detectable in the livers of homozygous mutants, and half normal activity was observed in heterozygous littermates. The heterozygous mice are healthy and develop normally, but homozygous mutants stop gaining weight at about 10 h after birth

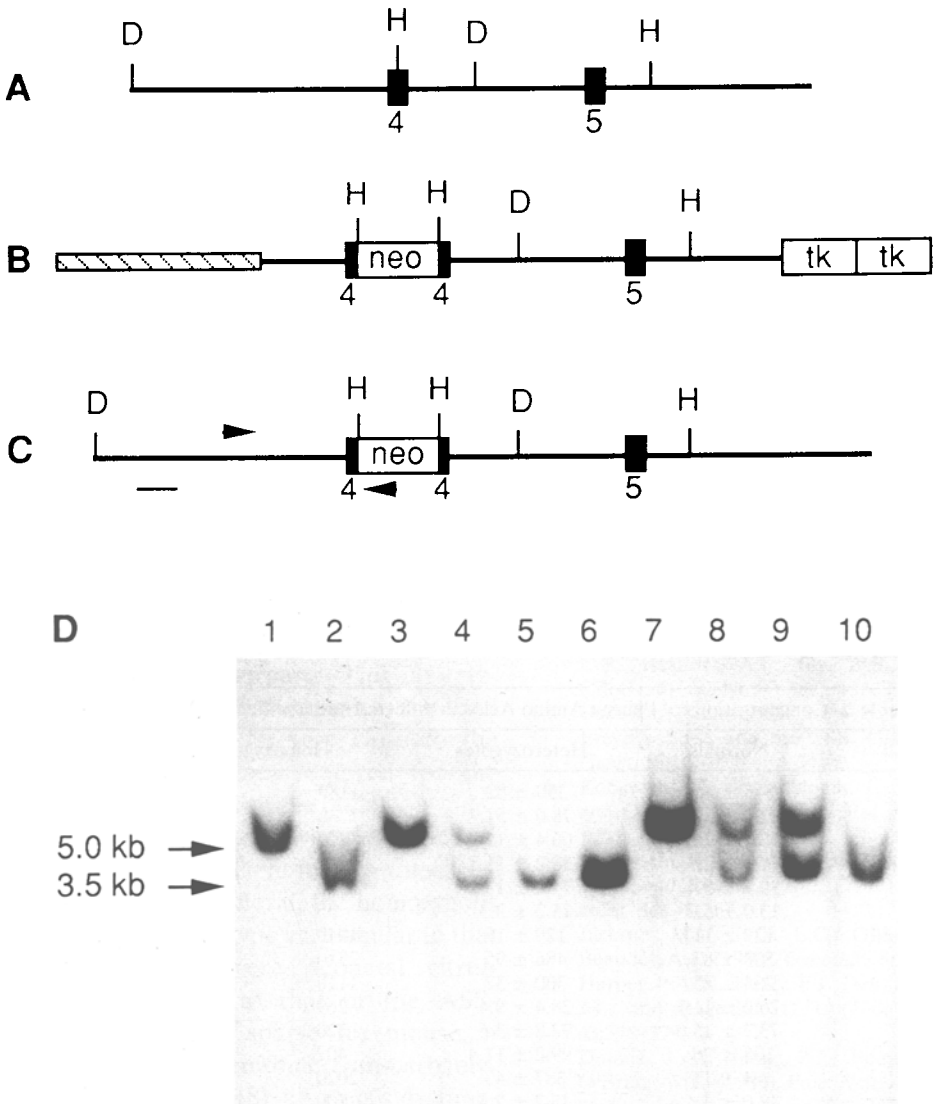

Fig. 1. Strategy for the preparation of the targeting construct and Southern blotting confirming the germ line transmission of the disrupted gene. (A) The region of the *Ass* gene to be disrupted. Exons 4 and 5 are shown as black boxes and HindIII and DraI sites are indicated. (B) The linearized targeting construct. A neomycin resistance cassette containing an RNA polymerase II promoter and bovine growth hormone polyadenylation sequence (11) was inserted into the HindIII site in exon four. Two copies of the herpes simplex virus thymidine kinase gene driven by the MC1 promoter were placed at the 3′ end. The hatched line represents the vector backbone of the construct. (C) Structure of the disrupted *Ass* gene. The positions of oligonucleotide primers used in the polymerase chain reaction to identify homologous recombinants are designated by the arrows. The thin line near the 5′ end shows the location of a 0.5-kb XbaI/EcoRI fragment used as a probe in Southern blotting. (D) Southern blot from the litter of a heterozygote × heterozygote mating. DNA was digested with DraI and hybridized using the XbaI/EcoRI probe. Lanes 2, 5, 6, and 10 show the 3.5-kb band expected from wild-type animals; lanes 1, 3, and 7 show the 5-kb band expected from homozygous mutants; and lanes 4, 8, and 9 show the pattern expected of heterozygotes.

Table 1. Argininosuccinate Synthetase Enzyme Activity in Neonatal Mouse Liver

Genotype	Number of animals	Activity (μmol/min/g tissue)[a]
Normal	3	2.66 ± 0.85
Heterozygotes	5	1.33 ± 0.27
Homozygotes	6	0.002 ± 0.003

[a]Activity is expressed as the mean ± one standard deviation.

and usually die before their 24th hour of life. We measured plasma ammonia levels in 16- to 19-h-old animals and found that homozygous mutants had an ammonia concentration of 2680 ± 970 μM (mean ± standard deviation of four animals), which is 16-fold higher than that of normal littermates. Histologic examination of the affected animals revealed no gross abnormalities (data not shown).

Blood Chemistry. Since the hallmark of ASS deficiency in humans is a markedly elevated level of blood citrulline, we measured plasma amino acids in a litter of mice born from a pair of intercrossed heterozygotes (Table 2). Blood was obtained 16 h after birth. As anticipated, the homozygous mutant mice had a significantly higher concentration of citrulline and a decrease in arginine, changes which are of the same order of magnitude as in human patients with citrullinemia (2). In addition, an overall two- to fourfold increase in almost all amino acids was found in the homozygous affected animals. We postulate that this may simply reflect a general deterioration in the metabolism of the animals due to the advanced state of the illness at the time they were sacrificed. This overall increase in amino acid levels is not seen in the urease-infused rat model of hyperammonemia (17); however, these studies were conducted on adult rats. One notable difference between the mice and

Table 2. Concentrations of Plasma Amino Acids in Affected and Unaffected Animals[a]

Amino acid	Normals	Heterozygotes	Homozygotes		Ratio
Alanine	345 ± 48	350 ± 82	1180	850	2.94
Arginine	82.0 ± 32.8	78.0 ± 51.7	36	33	0.42
Asparagine	68.7 ± 7.2	66.4 ± 11.7	279	243	3.80
Aspartic acid	31.3 ± 2.3	38.2 ± 11.5	871	1020	30.2
Citrulline	39.7 ± 8.4	74.2 ± 13.5	2560	2540	64.2
Cystine	13.0 ± 5.2	15.2 ± 3.3	55	58	4.35
Glutamic acid	127 ± 11	129 ± 19	1040	1050	8.23
Glutamine	503 ± 87	486 ± 95	2310	1850	4.14
Glycine	296 ± 23	300 ± 32	1110	1250	3.99
Histidine	26.0 ± 11.5	28.4 ± 9.4	269	188	8.79
Isoleucine	73.7 ± 15.0	72.8 ± 3.6	220	210	2.92
Leucine	105 ± 26	99.2 ± 11.4	402	353	3.60
Lysine	404 ± 111	387 ± 43	2020	1860	4.80
Methionine	48.0 ± 4.4	45.2 ± 7.5	109	85	2.02
Ornithine	52.3 ± 14.8	73.2 ± 15.5	164	170	3.19
Phenylalanine	82.0 ± 13.1	61.6 ± 13.9	139	85	1.37
Proline	205 ± 26	187 ± 35	685	610	3.16
Serine	257 ± 28	291 ± 43	628	555	2.30
Threonine	221 ± 33	231 ± 26	475	430	2.04
Tryptophan	59.7 ± 9.5	49.2 ± 6.5	141	125	2.23
Tyrosine	109 ± 19	102 ± 25	469	425	4.10
Valine	133 ± 27	128 ± 10	382	360	2.80

[a]A litter of 10 animals from a heterozygote × heterozygote mating were sacrificed at 16 h after birth. Individual data are provided for each of two homozygous mutant animals, while means and standard deviations for three wild-type and five heterozygous animals are given. Concentrations are given in micromoles per liter. The last column gives the ratio of the mean of the two homozygotes to the mean of the normals.

their human disease counterparts was that the homozygous mice showed a 30-fold increase in aspartic acid, while aspartic acid levels are not increased in humans with citrullinemia. This observation presumably reflects differences in the activities of transaminases in the liver of mice versus humans in the neonatal period.

Like their human counterparts, wild-type and heterozygous animals exhibited little disparity in their plasma amino acid levels with one exception. The citrulline level in heterozygous mice was 1.9-fold higher than in their homozygous normal littermates, producing a statistically significant ($P < 0.01$, Student's t test) difference. No such difference has been observed in humans or in cattle. Our ability to detect this in the mice may be due to the more nearly matched genotype at other loci (even though these animals are not inbred), producing a more narrow range of normal values as compared to their human counterparts. This observation implies that, in newborn mice, the level of ASS activity may be very close to the minimum catalytic level required for the maximum activity of the urea cycle.

In summary, we have developed a mouse line that is deficient for argininosuccinate synthetase. In general, the homozygous animals have a phenotype very similar to that of humans with severe neonatal citrullinemia, as well as to that of the ASS-deficient cattle. The sparse fur mouse, a naturally occurring ornithine transcarbamylase-deficient mouse (18), also has a dysfunctional urea cycle. These mice develop a milder hyperammonemia, and they are not as severely affected as the ASS-deficient mice. Stratford-Perricaudet et al. (19) have developed an adenovirus-based gene therapy for the sparse fur mouse that reduces the symptoms of these animals. We expect that a similar therapy could be developed for citrullinemia using our mouse as a model system. The citrullinemic mouse offers a major advantage for such studies, which is the ability to quantitate blood citrulline as a valuable index of therapeutic effect.

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