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N-Ethyl-*N*-nitrosourea-induced null mutation at the mouse *Car-2* locus: An animal model for human carbonic anhydrase II deficiency syndrome

(inborn error of metabolism/mutagenesis/osteopetrosis/renal tubular acidosis)

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ABSTRACT **Electrophoretic screening of (C57BL/6J × DBA/2J)F_1 progeny of male mice treated with *N*-ethyl-*N*-nitrosourea revealed a mouse that lacked the paternal carbonic anhydrase II (CA II). Breeding tests showed that this trait was heritable and due to a null mutation at the *Car-2* locus on chromosome 3. Like humans with the same inherited enzyme defect, animals homozygous for the new null allele are runted and have renal tubular acidosis. However, the prominent osteopetrosis found in humans with CA II deficiency could not be detected even in very old homozygous null mice. A molecular analysis of the deficient mice shows that the mutant gene is not deleted and is transcribed. The CA II protein, which is normally expressed in most tissues, could not be detected by immunodiffusion analysis in any tissues of the CA II-deficient mice, suggesting a nonsense or a missense mutation at the *Car-2* locus.**

In 1972, the first reports appeared of a new inborn error of metabolism in humans that was manifested as the combination of osteopetrosis and renal tubular acidosis (1–3). Intracranial calcifications and mental retardation were later reported in association with this disorder (4–6). On the basis of pedigree analysis, the inheritance pattern appeared to be that of an autosomal recessive mutation. Electrophoretic, chromatographic, and immunodiffusion studies performed on members of one of these families established that erythrocytes of the affected individuals were deficient in the carbonic anhydrase II (CA II) isozyme (7). Codominant inheritance of this enzyme deficiency was compatible with the recessive inheritance of the disease (7–10). However, the clinical heterogeneity of affected humans remains unexplained. The inability to study CA II levels in kidney and bone has prevented a firm demonstration of the relationship between CA II deficiency in these tissues and the clinical disorder.

A CA II-deficient animal model promises to be useful in confirming the relationship between the enzyme deficiency and the clinical phenotype. In general, animal models of human genetic disorders have a great potential for elucidating the underlying mechanisms of disease and for developing effective therapeutic strategies to be used with humans. A number of such models have resulted from spontaneous mutations in several animal species (11). A relatively recent development has been the induction of potentially useful mutations by using mutagens. As a result, mouse models of α-thalassemia (12, 13), osteogenesis imperfecta (14), polycythemia (15), and glucose phosphate isomerase deficiency resulting in macrocytic anemia‡ have been reported.

The CA II gene of the mouse has been characterized, and its protein product is known to express genetic variability (16, 17). In mice, the genes for CA I and CA II (*Car-1* and *Car-2*) are closely linked at the centromeric region of chromosome 3 (17), and several allelic electrophoretic variants have been described at both loci; two of the CA II alleles, *Car-2^a* and *Car-2^b*, are characteristic of C57BL/6J and DBA/2J mice, respectively (17).

In this report, we describe a null mutation of the *Car-2^b* allele of a male DBA/2J mouse induced by the mutagen *N*-ethyl-*N*-nitrosourea (EtNU). Homozygotes for the new allele have a syndrome with some features of the human CA II deficiency.

MATERIALS AND METHODS

The mouse *Car-2* null mutant was produced in a mutagenesis program in which a dose of EtNU at 200 mg/kg was administered to DBA/2J male mice. Methods for dosing of animals with EtNU have been described (18, 19).

The DBA/2J males were mated to C57BL/6J females 10 weeks or more after treatment to obtain progeny from germ cells that were exposed to EtNU as spermatogonia. Blood and kidney samples from the (C57BL/6J × DBA/2J)F_1 progeny were screened for variations at 32 loci by starch gel electrophoresis and broad-range isoelectric focusing, as described elsewhere (18, 19). CA II in F_1 animals appears as two bands (i.e., CA IIa and CA IIb) on the broad-range focusing gels.

The original F_1 mutant was backcrossed to C57BL/6J females. Intercrosses of carriers and additional backcrosses were also performed. The CA II phenotypes of all progeny were determined in blood samples by broad-range isoelectric focusing (19) or by electrophoresis on cellulose acetate (Cellogel, Whatman) at pH 5.4.

Developmental curves were generated by weighing the mice several times a week. Their CA II phenotypes were determined after weaning.

Urine pH values were determined on the droplets voided when the mice were handled, using ColorpHast pH reagent strips accurate to ±0.25 pH unit. Urine pH values were recorded while the mice were on a regular diet (Ralston Purina Lab Chow) with either tap water or various concentrations of NH_4Cl administered in the drinking water to test their ability to adapt to an acid load (20). Blood gases were studied by standard clinical laboratory procedures using

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Abbreviations: EtNU, *N*-ethyl-*N*-nitrosourea; CA, carbonic anhydrase; PGK, phosphoglycerate kinase.

‡Bhattacharjee, D., Charles, D. S. & Pretsch, W., Fourth International Conference on Environmental Mutagens, June 24–28, 1985, Stockholm, p. 101 (abstr.).

heparinized blood obtained by cardiac puncture of anesthetized mice.

The presence of CA II proteins in various tissues was determined by immunodiffusion analysis. Tissues from normal and deficient animals were homogenized in 0.9% NaCl (1 g/ml) with a ground glass homogenizer; insoluble material was removed by centrifugation. Goat antisera prepared against mouse CA I or CA II were placed in the center wells of Ouchterlony plates, and the tissue homogenates and controls were placed in the surrounding wells. The plates were developed at 37°C for 3–7 hr.

Southern blots were prepared by standard techniques (21). Total RNA was isolated by the guanidine isothiocyanate procedure (22), and the technique for slot blots was adapted from standard techniques (21). The CA II probe used for RNA transfer blotting and Southern analyses were pMCAII (17). The phosphoglycerate kinase-1 (PGK-1) cDNA probe used as a control was pHPGK-7e (23). Probes were radiolabeled by the random primer labeling method (24).

RESULTS

A (C57BL/6J × DBA/2J)F_1 male mouse was identified as a variant by the absence of the band specified by the *Car-2*^{b} allele (Fig. 1) inherited from the DBA/2J male parent. The band specified by the *Car-2*^{a} allele of the female parent was in the normal position on the focusing gel. All other loci screened in the blood and kidney samples from this animal were normal. The 32-locus electrophoretic profiles of the parents and the 6 siblings and 25 half-siblings of the mutant were entirely normal. In particular, the *Car-2* bands were in the position appropriate for each strain.

The variant animal was backcrossed to C57BL/6J females. Female progeny from this backcross, presumably *Car-2*^{a}/*Car-2*^{n} because of light *Car-2*^{a} bands, were mated with the original F_1 mutant and somewhat less than the predicted one-fourth of their progeny lacked any bands in the *Car-2* region (Table 1). In all crosses performed, the new mutation behaved as a Mendelian alternate at the *Car-2* locus with a deficiency of homozygous nulls by the time of weaning, which seems to correlate with deaths of severely runted pups in the litters at risk.

Specific antibodies to mouse CA II (and to CA I as a control) were used to study CA II protein levels in dissected organs. Erythrocytes of the deficient animals were similar to those of the human patients in showing no detectable CA II and normal levels of CA I (Fig. 2*A*). When a variety of tissues (i.e., kidney, stomach, brain, and large intestine) were compared between homozygous null and sibling wild-type mice for CA II antigen, no CA II was detected in the nulls (Fig. 2 *A* and *B*). When these same tissues were tested for the CA I antigen, no differences were noted between the null homozygotes and their normal siblings (Fig. 2*B*).

Quantitation of CA II mRNA by slot-blot analysis showed some variability in the CA II mRNA levels in a number of tissues of CA II-deficient mice (Fig. 3*a*). Hybridization of the same slot blot with a human cDNA clone for the housekeeping enzyme, PGK-1, which, in the testes, also hybridized to PGK-2, demonstrated that the ratio of hybridization with the CA II probe to the hybridization with the PGK-1 probe was the same in the slots from deficient mice and the sibling controls (not shown).

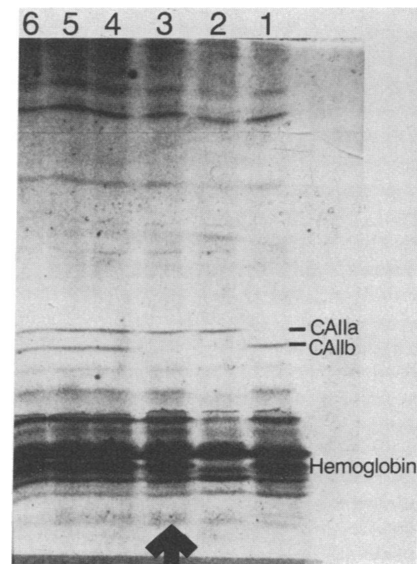

FIG. 1. Isoelectric focusing patterns of CA II phenotypes (CA IIa and CA IIb) of male DBA/2J mouse (*Car-2*^{b}/*Car-2*^{b}) (lane 1), female C57BL/6J mouse (*Car-2*^{a}/*Car-2*^{a}) (lane 2), CA IIb-deficient F_1 mouse (lane 3), and F_1 offspring (*Car-2*^{a}/*Car-2*^{b}) of C57BL/6J × DBA/2J (lanes 4–6).

Southern analyses of genomic DNA from the null homozygotes did not demonstrate any major deletions or detectable alterations of restriction fragment length sizes with a variety of restriction enzymes (Fig. 3*b* provides one example).

The CA II-deficient mice have very different growth curves than their sibs (Fig. 4), being significantly smaller than normal siblings of the same sex. Affected males eventually achieve weights comparable to those of their normal sisters but they are significantly smaller than their normal brothers.

When given tap water to drink, the urine of the deficient mice has a more alkaline pH than that of their wild-type sibs (Table 2). Mice, like rats (20), can tolerate large amounts of NH_4Cl in their drinking water without an alteration in urine pH. The fact that neither the CA II-deficient mice nor their heterozygous sibs show a significantly lower urine pH after ingesting NH_4Cl is consistent with these findings.

The null homozygotes were less tolerant of NH_4Cl in the drinking water than normal sibs. The small sample size of null homozygotes studied is due to the extreme difficulty of obtaining any urine at all from them during the NH_4Cl regimen. These mice looked ill, and one death occurred in

Table 1. Crosses involving *Car-2*^{n} carrier mice

Cross	No. of litters	*Car-2* genotype of progeny: *a/a* or *a/n**	*a/n*	*b/n*	*a/b*	*n/n*	Total progeny typed
Backcross of carrier daughters to original mutant							
Car-2^a/*Car-2*^{n} × *Car-2*^{a}/*Car-2*^{n}	5	24				4	28
Car-2^b/*Car-2*^{n} × *Car-2*^{a}/*Car-2*^{n}	10		10	16	18	6	50
Carrier crossed to DBA/2J mice							
Car-2^a/*Car-2*^{n} × *Car-2*^{b}/*Car-2*^{b}	15			36	39		75

*Although some heterozygous animals can be identified by light CA II bands, the heterozygous null animals and homozygotes cannot be distinguished from each other in all cases.

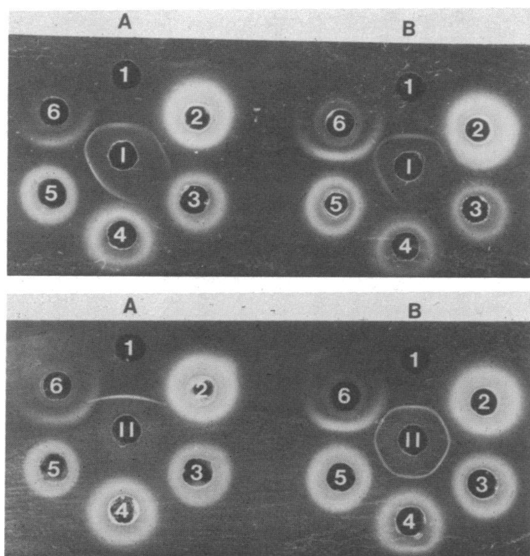

FIG. 2. Ouchterlony immunodiffusion patterns of CA I and CA II in CA II-deficient mutant mice (*A*) and their sibling controls (*B*). Homogenates from wells 2–6 are kidney, stomach, brain, large intestine, and erythrocytes, respectively. Center wells contain anti-mouse CA I (I) and CA II (II) antisera. Wells A1 and B1 contain purified mouse CA I and CA II controls, respectively.

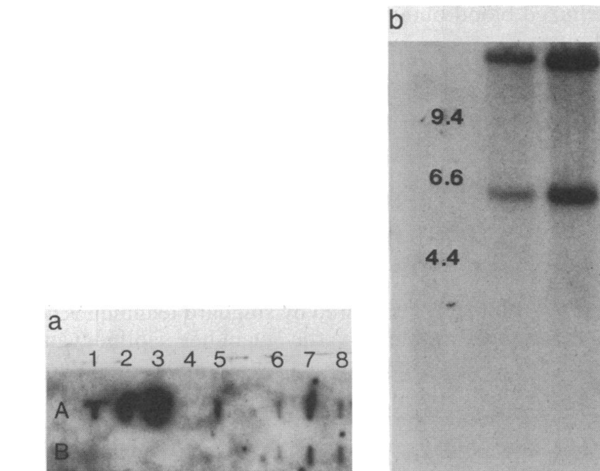

FIG. 3. (*a*) Slot blot of RNA from mouse CA II nulls and controls. Slots 1A–3A contain 1, 10, and 100 pg of mouse CA II cDNA, respectively, from the insert of pMCAII (16). The remaining slots all contain 1 μg of nucleic acid: 4A, yeast RNA; 5A, DBA/2J DNA; 6A, control spleen RNA; 6B, null spleen RNA; 7A, control small intestine RNA; 7B, null small intestine RNA; 8A, control testes RNA; 8B, null testes RNA. The slot was probed with the same mouse CA II cDNA used in slots 1A–3A after radioactive labeling. (*b*) Southern analysis of CA II null (left) and control DNA (right). DNA was digested with *Bam*HI, electrophoresed, transferred to GeneScreen*Plus* by the method of Southern (25), and hybridized with the probe described in *a*. Size markers are in kilobases.

the 0.9% NH_4Cl group before mice were taken off ammonium chloride. When provided with the higher doses of NH_4Cl the deficient mice rapidly lost weight and did not look well while their normal sibs dealt successfully with the acid load. No urine at all could be obtained from homozygous null mice treated with 1.2% and 1.5% NH_4Cl.

Blood gases were measured on cardiac blood from anesthetized animals. Blood pH varied widely because of variations in the induced hypoxia but blood bicarbonate was only 18.25 ± 0.43 mEq/liter in six CA II-deficient mice, whereas it was 22.18 ± 0.35 mEq/liter in six sibling controls (t = 7.11, $P < 0.001$).

Skeletal x-rays of CA II-deficient mice and their sibs

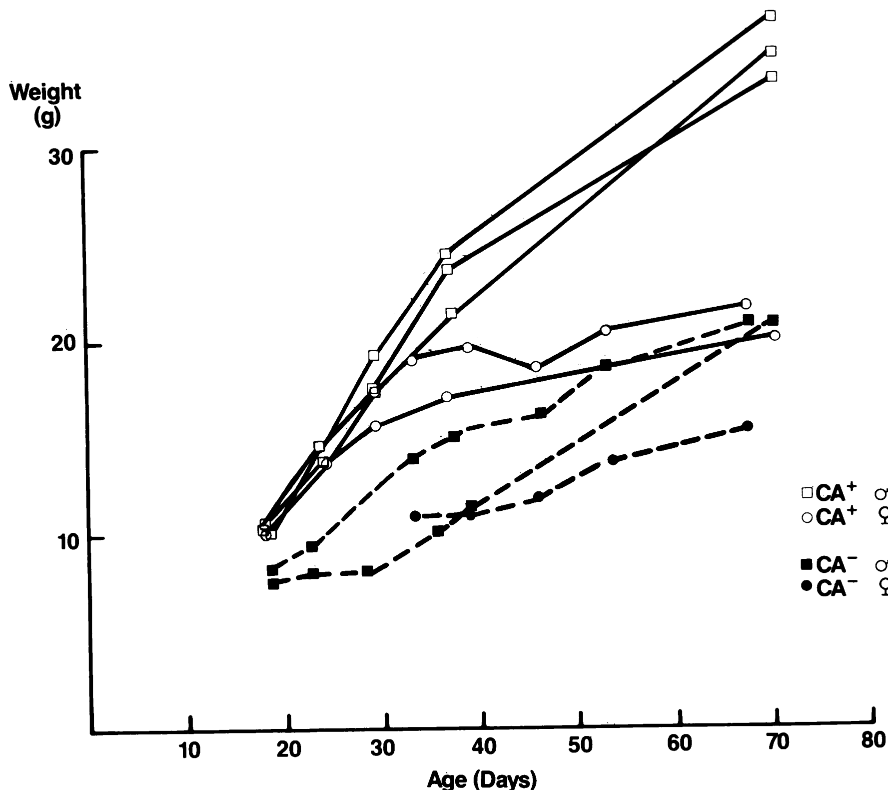

FIG. 4. Growth curves of CA II-deficient mice and their sibling controls. ■, CA II-deficient males; ●, CA II-deficient females; □, normal males; ○, normal females.

Table 2. Effect of NH_4Cl ingestion on urinary pH in CA II-deficient mice and sibling controls

NH_4Cl added to regular diet, %	Sibling control*		CA II-deficient mice†		
	n	Mean	*n*	Mean	*P*
None	21‡	6.0 ± 0.2	8	6.9 ± 0.3	<0.001
0.3	4	6.0 ± 0	3	7.0 ± 0.1	<0.001
0.6	6	5.8 ± 0	4	6.6 ± 0.4	<0.001
0.9	6	5.8 ± 0.2	2§	6.9 ± 0.1	<0.001
1.2	5	5.8 ± 0.2	0¶	—	—
1.5	4	5.9 ± 0.1	0¶	—	—

Data are presented as mean ± SD.
*Genotypes: $Car\text{-}2^a/Car\text{-}2^b$ and $Car\text{-}2^a/Car\text{-}2^n$.
†Genotype: $Car\text{-}2^n/Car\text{-}2^n$.
‡Seven males (6.0 ± 0.2) and 14 females (6.0 ± 0.2).
§One death.
¶No urine obtainable on mice in these groups.

disclosed no signs suggestive of osteopetrosis even in animals as old as 15 months (Fig. 5). Although the skeleton is much smaller in the homozygous null mice (as expected from their decreased weight), marrow cavities are equally visible and seem proportional in size in the deficient and nondeficient mice. In addition, no brain or renal calcifications are noted by x-ray at 15 months of age in these mice. The lack of brain calcification has been confirmed by histological studies (M. S. Ghandour, personal communication).

DISCUSSION

A mutation screening project for EtNU-induced mutations revealed a new mutation at the *Car-2* locus that creates a pathological state with features of the human deficiency. The mutation must be *de novo* since both parents and all of the siblings of the original mutant had normal CA II patterns. This mutation is most likely to have been induced with EtNU, as the *Car-2* allele from the treated father is affected.

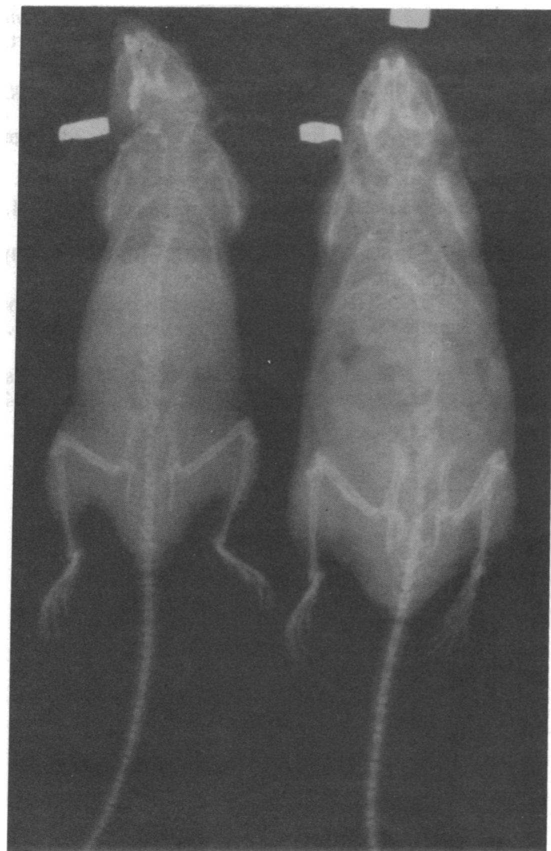

FIG. 5. Skeletal x-rays of a 15-month-old, CA II-deficient mouse (*Left*) and an unaffected 14-month-old sibling (next litter) control (*Right*).

All mammalian EtNU mutations sequenced to date have been found to result from single base-pair substitutions (‡, 26–28). Lack of any rearrangements in the CA II gene detectable by Southern blotting is consistent with this mode of action of EtNU. Null mutations, including the one reported here, comprise a significant proportion of mutations induced by EtNU (29).

The induction of a null allele by an agent causing point mutations could be due to a number of mechanisms. It is possible that the mutation in the CA II gene of these mice resulted in a nonsense codon causing premature termination of the translated protein. A missense codon resulting in a full-length, but rapidly degraded, protein could also result in a null mutation that is Crm^-, such as this one.

Most of the electrophoretically detected EtNU-induced null alleles fail to show discernable physiological effects in the homozygote (29), with the significant exception of glucose phosphate isomerase (ref. 28; S.E.L., unpublished data) and CA II described in this study. Animals without detectable CA II activity are viable but are seriously defective physiologically. They are smaller than their siblings of the same sex, starting not later than at 2 weeks of age. This is presumably a consequence of their renal tubular acidosis and is analogous to the human disorder in which growth is normal during infancy, but short stature and failure to thrive appear from early to middle childhood (7, 8).

The primary difference between the syndromes in mouse and in man is the development of osteopetrosis in affected humans; there is no sign of this even in old mice. The lack of osteopetrosis may be due to the absence of a Haversian system as has been observed in rats (30). Apparently rodents do not remodel bone the way humans do—i.e., their remodeling is limited to the surfaces of their smaller bones and does not occur internally. Thus, the lack of osteopetrosis in CA II-deficient mice, as compared to CA II-deficient humans, suggests the possibility that the function of CA II in osteoclasts is essential for internal remodeling of bone and not for its surface deposition. Alternatively, mice may have enzymatic or physiological pathways involved in remodeling skeletal bone different from humans, in which CA II seems to be important. In fact, osteopetrosis is known to exist in microphthalmic (*mi/mi*) mice but levels of CA II are normal in erythrocytes and osteoclasts of such osteopetrotic (*mi/mi*) mice (31).

The apparent lack of brain calcification in *Car-2* homozygous null mice may be due to the relative difference in life spans of mice and humans or it may reflect a species difference for the physiological roles of CA II in maintaining acid–base balance in the brain. CA II is normally expressed in a wide variety of mouse tissues, compared with the seemingly more limited distribution of the CA I and CA III isozymes (32, 33). In CA II-deficient humans, dysfunctions of bone (osteopetrosis) and brain (cerebral calcification) are evident; nevertheless, no evidence of abnormal function was observed in other human tissues (7), which presumably also lack CA II as do CA II-deficient mice. One possible explanation for the viability of individuals lacking CA II is that normally other isozymes of CA (e.g., CA I, CA III) may be expressed together with CA II in certain cells and that they can substitute for CA II in its absence. This has already been demonstrated in CA II-deficient humans, in which the CA I isozyme appears to be capable of carrying out the erythrocyte function of CA in the absence of the CA II isozyme (34).

The mutant null homozygous mice serve as models for the analysis of CA gene expression in mammals. The specific

defect provides an opportunity to study the genetic regulation of the *Car-2* locus. The null mutation also facilitates the study of tissue-specific expression of the other CA isozymes in the absence of the more abundant CA II isozyme. The availability of this mouse model for human CA II deficiency may allow clarification of several questions about the human disease. Responses to variations in diet and medication for renal tubular acidosis in treated CA II-deficient mice may help to determine whether the clinical heterogeneity seen in humans may have an environmental component. New or alternative modes of therapy for osteopetrosis and renal tubular acidosis may be evaluated based on these findings. Finally, CA II-deficient mice can be made transgenic for human or mouse CA II genes and thus evaluated for correction of the abnormal phenotype.

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