PheNylketONuRia (PKU) in humans results from a deficiency in phenylalanine hydroxylase (PAH) (SCRIVER and CLOW 1980). When untreated, this condition results in severe and irreversible mental retardation. Treatment consists of careful regulation of essential dietary phenylalanine (PHE) sufficient to provide for protein synthesis without excess accumulation. With precise dietary control, initiated shortly after birth, the detrimental effects of PKU can largely be avoided. Since these effects most severely impact the developing central nervous system, PKU patients who are weaned from the PHE-controlled diet in later childhood, although recent evidence suggests that diet termination may have harmful effects (MONTAL and MICHAELS 1991). In addition to the effects of PKU on the affected individual, teratogenic effects result when pregnancy in a woman with PKU is not treated with the PHE-restricted diet. In this situation, called maternal PKU, the developing heterozygous fetus is likely to be severely compromised: a high incidence of microcephaly, mental retardation, congenital heart defects, and other abnormalities are observed (LEVY and WAISBREN 1983). By contrast, strict regulation of PHE intake from the onset of pregnancy allows normal fetal development (SCRIVER, KAUFMAN and WOO 1989; LENKE and LEVY 1982).

The biochemistry and genetics of PKU are well understood. PKU patients display at least 20-fold elevated serum PHE and usually <1% activity of PAH [McKusick no. 26160; EC number 1.14.16.1 (KAUFMAN et al. 1975)]. The genetic locus encoding this enzyme has been mapped, and the PAH coding sequence has been cloned in both human (WOO et al. 1983) and mouse (KWOK et al. 1985). Specific DNA alterations have been found for many human PKU mutations (LEDLEY et al. 1990). Despite these advances, little is known about certain aspects of the biology of PKU. Why is PAH deficiency so damaging to the developing brain? Is PHE the ultimate inhibitor of postnatal central nervous system development and the teratogen in maternal PKU (GUTTLER et al. 1987; LEVY 1988)? Can methods for somatic gene therapy be developed? Clearly, an animal model would be invaluable in studies aimed at addressing these questions.

For some time attempts have been made to produce rat PKU models by administering PHE analogs that inhibit PAH activity (FIGLEWICZ and DRUSE 1980; HUETHER and NEUHOFF 1981; LANE et al. 1980). However, the interpretation of such experiments is complicated by side effects of the inhibitors. Thus, a genetic animal model with the same defect as in the human PKU patient is more appropriate for studying the biology of PKU.

We have reported germline ethylnitrosourea (ENU) mutagenesis followed by a PHE clearance screen to isolate a mouse mutant line, PAH<sup>hph<sup>-</sup></sup>, deficient in PAH activity (McDONALD et al. 1990). This first rodent mutant fails to exhibit the biological effects associated with human PKU. For clarity and consistency of nomenclature, we are renaming this line PAH<sup>enu1</sup> (enu1). Subsequent mutant alleles, also induced by ENU, will be PAH<sup>enu2</sup>, PAH<sup>enu3</sup>, etc. The present communication describes the results of a second round of mutational analysis, with the consequent isolation of two new mutant alleles of Pah. The new lines display...
numerous biological characteristics of human PKU and illustrate one way of perfecting mouse models.

MATERIALS AND METHODS

**Mice and diets:** Animals were maintained and bred by standard methods of mouse husbandry (LES 1966) using Teklad Breeder Blox containing 20% protein. The PHE deficient diet was Teklad Research Diet #TD90363, containing all essential amino acids except PHE; with sucrose (490.88 g/kg); corn starch (150 g/kg); corn oil (100 g/kg); cellulose fiber (50 g/kg); mineral mix, AIN-76 (55 g/kg); calcium phosphate, dibasic (4.5 g/kg); vitamin mix, Teklad 40060 (10 g/kg); and ethoxyquin as an antioxidant (0.02 g/kg). The BTBR strain has been described (SHELDYlovsky et al. 1986).

**Biochemical determinations:** Serum was prepared from blood collected from the retroorbital sinus into heparinized capillary tubes. PHE concentrations were determined by a fluorometric assay, described in Sigma Diagnostics Procedure No. 60-F (McCaman and Robins 1962). Urinary ketones (level of detection 15 mg/dl) were measured by the color produced by a drop of urine on Phenistix Reagent Strips (Miles Inc. Diagnostic Division, Elkhart, Indiana 46515).

**Phenylalanine hydroxylase assays:** Crude liver lysates were prepared and assayed as previously described (McDonald et al. 1990). In brief, tissue was homogenized in 150 mM KCI/0.7 mM 2-mercaptoethanol (pH 7.0) and clarified by centrifugation at 14,000 x g in the cold for 15 min. PAH activity was measured by the phenylalanine-dependent oxidation of NADH in excess quinonoid dihyropteridine reductase and 6-methyltetrahydrobiopterin. The rate of oxidation was linear for at least 15 min and was directly proportional to total protein concentration over a range of at least 0.6–1.4 mg/ml reaction volume. The activities presented are the total values minus the background rate ± standard deviation. Each value is the average of at least four determinations.

**Protein:** Concentrations were determined by the Bioxiet method.

**Western blot analysis:** Flash frozen liver portions, about 100 mg wet weight, were homogenized on ice in 2 ml RIPA buffer (Sutrave, Kelly and Hughes 1990) containing the protease inhibitors aprotinin and phenylmethylsulfonyl fluoride. Samples were electrophoresed at 6 µg/lane in a 15% SDS/polyacrylamide gel (PAGE), with 0.25 µg rat PAH (Sigma) as control, and immunoblotted as described by Thompson et al. (1989).

**RNAse protection assay:** Total liver RNA was isolated (Chomozynski and Sacchi 1987) from flash frozen livers. The PAH cDNA was cloned into a pGEM7ZF(+) vector and digested with the restriction endonuclease, DraI. 5′-P-labeled PAH riboprobe was prepared by transcription from the 3′-end of the cDNA to yield a 358-bp fragment. Assays were carried out at 37° as described by Greenberg (1987) with only ribonuclease T1. The nuclease-protected fragment is expected to be 315 bp.

RESULTS

**Isolation of PAH mutants:** Inbred BTBR males were treated with the germline mutagenENU and mated to normal BTBR females, as previously described (LES 1966). Three hundred and fifty progeny of this cross were each screened as follows for mutations that fail to complement Pahmnu. The potential carrier was mated to a Pahmut homozygote, and neonates were tested for hyperphenylalaninemia by the Guthrie test (Guthrie and Susi 1963). When a proportion of the test progeny was found to be hyperphenylalaninemic, the carrier parent was mated with a normal BTBR partner, and a congenic mutant line was subsequently established. We previously found (unpublished) that neonates homozygous for Pahmnu display a very slight Guthrie-positive phenotype. Using the present strategy, we hoped to identify new mutations more severely affected and displaying a PKU phenotype. Among the 350 potential carriers, each representing one mutagenized paternal gamete, we isolated two new alleles, Pahmnu2 and Pahmnu3, each with more pronounced phenotypes than the original.

Noncomplementation does not itself prove allelism; cases have recently been described in which recessive alleles at distinct loci fail to complement (see Stearns and Botstein 1988). Indeed, the high frequency of recovery of these mutations [nearly 10 times that observed per locus in other murine genes (Russell et al. 1979)] raises the question of whether they are each alleles of Pahmnu. Tight genetic linkage was demonstrated by scoring the phenotype of progeny obtained by intercrossing F1 mice doubly heterozygous for both Pahmnu and either Pahmnu2 or Pahmnu3. At 5 days of age a drop of tail blood was tested for hyperphenylalaninemia with the Guthrie assay (Guthrie and Susi 1963). In questionable cases, the mutant phenotype was confirmed at weaning with a PHE clearance test (McDonald et al. 1990). Among over 100 progeny from each of these two crosses, all exhibit a mutant phenotype. If these noncomplementing mutations were unlinked to Pahmnu, one would expect a normal phenotype in 5/16 of the progeny. Since none were observed, both of these mutations map within 5.6 cM from each other.

Further, in crude liver extracts, PAH activity was markedly deficient in each mutant animal (Table 2). Mixing experiments (not shown) demonstrated that mutant extracts do not contain an inhibitor of PAH activity. Western blot analysis, with a polyclonal anti-PAH antiserum (Friedman, Lloyd and Kaufman...
TABLE 1
Serum PHE and urinary ketone concentrations in adult wild-type and mutant strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. of animals</th>
<th>[PHE]</th>
<th>[KETONE]</th>
</tr>
</thead>
<tbody>
<tr>
<td>On Breeder Blox (20% protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>20</td>
<td>1.5 ± 0.5</td>
<td>neg (2)</td>
</tr>
<tr>
<td>enu1</td>
<td>50</td>
<td>2.8 ± 1.0</td>
<td>neg (1), 15 (2)</td>
</tr>
<tr>
<td>enu2</td>
<td>100</td>
<td>23.1 ± 6.9</td>
<td>15–40 (2), 100 (1)*</td>
</tr>
<tr>
<td>enu3</td>
<td>10</td>
<td>20.3 ± 5.9</td>
<td>15–40 (3), 100 (3)*</td>
</tr>
<tr>
<td>On defined diet supplemented with 100–125 mg/dl PHE in the drinking water</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>enu2</td>
<td>15</td>
<td>8.0 ± 6.6</td>
<td>15–40 (2)</td>
</tr>
<tr>
<td>enu3</td>
<td>2</td>
<td>7.1 ± 4.7</td>
<td>Not tested</td>
</tr>
</tbody>
</table>

PHE concentrations reported are the average, ± the standard deviation, of animals maintained on the indicated diet for at least 2 weeks prior to sampling. Values reported are all mg/dl. The number in parentheses indicates the number of animals tested.

* This value is significantly different from wild type (P < 0.0005, Wilcoxon rank sum test).

† In addition, these have been shown to contain elevated levels of phenyl lactic and phenyl pyruvic acids (J. WOLFF, unpublished).

TABLE 2
PAH activity in wild-type and mutant liver extracts

<table>
<thead>
<tr>
<th>Strain</th>
<th>Liver PAH activity (Δ O.D. × 107/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>40 ± 6</td>
</tr>
<tr>
<td>enu1</td>
<td>5 ± 5</td>
</tr>
<tr>
<td>enu2</td>
<td>0 ± 2</td>
</tr>
<tr>
<td>enu3</td>
<td>5 ± 5</td>
</tr>
</tbody>
</table>

Protein: PAH + e1 + e2 + e3 + enu1 + enu2 + enu3 + PAH
Lanes: 1 2 3 4 5 6 7 8

![Western blot analysis of crude liver lysates of wild type and mutants. PAGE analysis for PAH cross-reactive proteins was carried out on standard concentrations (6 μg) crude liver lysates of wild type (+) and the three HPH mutants: enu1 (e1), enu2 (e2), enu3 (e3). Lanes 1 and 8 contained 10 μg rat liver PAH (Sigma). Control lanes 1–3 were developed without anti-PAH antibody. Lanes 4–8 were developed after treatment with polyclonal sheep anti-rat PAH antibody (FRIEDMAN, LLOYD and KAUFMAN 1972) followed by alkaline phosphatase conjugated rabbit anti-sheep IgG. Bands were developed by incubating the immunoblot with alkaline phosphatase substrate.](image)

Maternal effect: In contrast to enu1, enu2 and enu3 females exhibited a severe maternal effect under normal husbandry. On our standard breeding diet (see MATERIALS AND METHODS) (about 1% PHE), the average litter size was close to normal, but no pups survived beyond several hours (Table 3). Like maternal PKU in humans, this effect depended entirely upon the genotype of the mother. Therefore, we have pooled data from crosses in which the paternal genotype was varied.

To find a diet permissive for breeding the new mutants as homozygotes, we started with a solid defined synthetic diet lacking PHE, which has been added to the drinking water at different concentrations. At 125 mg/dl PHE, the serum PHE levels and...
TABLE 3
Maternal effect of enu2 and enu3

<table>
<thead>
<tr>
<th>Maternal genotype</th>
<th>+/+</th>
<th>PahnY2/+</th>
<th>PahnY2/PahnY2</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of litters</td>
<td>48</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>No. of progeny at Birth</td>
<td>365</td>
<td>110</td>
<td>32a</td>
</tr>
<tr>
<td>Weaning</td>
<td>347</td>
<td>98</td>
<td>0</td>
</tr>
</tbody>
</table>

On defined diet supplemented with 100–125 mg/dl PHE in the drinking water

<table>
<thead>
<tr>
<th>Maternal genotype</th>
<th>+/+</th>
<th>PahnY2/+</th>
<th>PahnY2/PahnY2</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of litters</td>
<td>10</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>No. of progeny at Birth</td>
<td>39</td>
<td>47</td>
<td>8</td>
</tr>
<tr>
<td>Weaning</td>
<td>16</td>
<td>18</td>
<td>3</td>
</tr>
</tbody>
</table>

Matings were set up after females had been maintained on the indicated diet for at least 1 week. At weekly intervals females were palpated to detect pregnancies. Close to the expected end of the gestation period, females were checked daily for litters. The number of progeny were counted within 12 hr of birth and again at weaning.

a Total from six litters observed at birth.

TABLE 4
Effects of maternal genotype and diet on progeny survival

<table>
<thead>
<tr>
<th>Maternal genotype</th>
<th>+/+</th>
<th>PahnY2/+</th>
<th>PahnY2/PahnY2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestation:</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Postnatal</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>No. of litters</td>
<td>9</td>
<td>9</td>
<td>12</td>
</tr>
<tr>
<td>No. of progeny at Birth</td>
<td>55</td>
<td>40</td>
<td>86</td>
</tr>
<tr>
<td>Weaning</td>
<td>54</td>
<td>9a</td>
<td>80</td>
</tr>
</tbody>
</table>

On defined diet as in Table 3, during gestation and switched to standard diet within 12 hr of birth. Column (1), normal pups with wild type mothers; column (2), pups with enu2 mothers; column (3), pups from enu2 mothers, fostered to wild type within 12 hr of birth.

a Progeny from only two litters.

urinary ketones are only slightly elevated (Table 1) and hypopigmentation is partially reversed. This last effect is similar to that observed in human PKU patients on low protein diets (Bickel, Gerrard and Hickmans 1954; Woolf, Griffiths and Moncrieff 1955). Under these conditions, the mutant and wild type display similar breeding performances (Table 3). However, in each case, neonates are gradually lost during the first week until an average litter size of about two remains. If the wild-type mother is switched to the standard diet on the day of birth, progeny survival is normal (Table 4). The results obtained with litters from mutant mothers (Table 4) are dramatically different: progeny from only two of nine litters survived. By contrast, if progeny from mutant mothers are fostered at birth to wild-type mothers (on standard diet), survival is essentially normal.

We wished to test the effect of standard diet on prenatal development in mutant mothers. In addition, we wanted to know if progeny genotype was relevant to any detected dietary effects. Therefore, mutant females were bred to carrier males (on standard diet) and examined daily for vaginal coital plugs. On the 19th day of gestation pregnant females were sacrificed, pups were removed and fostered to wild-type mothers on standard diet. Survival, to weaning age, of pups from control, wild-type females, was about 90% (45 of 54), see Table 5. By contrast, survival from mutant females was about 20% (17 of 92) with no effect of progeny genotype.

DISCUSSION

The major result that we report is the establishment of two mutant mouse strains that each closely simulate human PKU. PAH enzyme is much reduced, and blood PHE and urinary ketones are correspondingly increased unless the dietary PHE intake is restricted. We observe evidence for abnormal central nervous system development, and will seek an objective measure of this effect. The mutants exhibit a severe maternal effect on standard breeding diet. We have not determined the cause of neonatal death and, therefore, we are not yet in a position to evaluate how closely these maternal effects reflect those described in human maternal PKU. However, this lethal effect is avoided when dietary PHE is low during gestation and normal from birth. Further studies are in progress to explore the effects on development of the pre- and postnatal metabolic environment.

The enu1 strain was selected on the basis of its inability to clear a load of injected PHE. Since we have not observed any other mutant phenotype in vivo, we conclude that the mutation is "leaky." Therefore, it was surprising to find that crude liver extracts of enu1 animals contained very little PAH enzymatic activity or CRM. Perhaps these animals make an altered enzyme that is rapidly degraded in vitro but more stable in vivo, resulting in sufficient catalytic
activity to catabolize the PHE provided in the standard diet.

Overall, we find no correlation between the amount of PAH-CRM and the mutant phenotype in the whole animal. In addition, the amounts of PAH-CRM and mRNA vary independently of each other. We find that enu2, the mutant with the most PAH-CRM, contains the lowest level of mRNA. Perhaps these unexpected results will be better understood when the molecular nature of each mutation within the PaK locus has been determined.

The mutants described here provide material that is necessary to study several aspects of PKU biology. We assume that it was purely fortuitous that the initial PAH mutant isolate was less suitable as a PKU model than the two new alleles. However, this experience underlines considerations that may be crucial in other mouse germine mutagenesis experiments. To obtain a broad spectrum of recessive mutant alleles at a locus, a two-step approach is most effective. The first step, generally requiring a three-generation pedigree screen, is logistically demanding. But the second step, a noncomplementation or "locus-specific" screen (Russell 1951; Lyon, Phillips and Bailey 1972), is more facile. In the absence of a holding generation, a mutagenized gamete is tested by a single testcross animal; when a holding generation is desired, as described here, a mutagenized gamete can be tested by one litter. Thus, both time and space are dramatically reduced compared with the three-generation scheme. These considerations are particularly important for genes with vital function. By necessity, the initial recessive allele must be "leaky" to permit viability. The second stage then identifies mutant alleles that may include recessive lethals. By contrast, the vital gene can first be mutated by gene targeting (for example, see Tybulewicz et al. 1992) and the null mutant allele propagated in heterozygous form. Next, mutagen-induced noncomplementing viable alleles can be readily isolated by a specific locus screen. Such a strategy may yield mouse models that accurately reflect the human disease phenotype (Wilson and Collins 1992; Davies 1992).

Because of their strong phenotype, these mutants can be directly utilized in exploring methods of somatic gene therapy. Since the mutations are congenic on an inbred strain of mice, one could directly introduce wild-type hepatocytes into mutants and assess correction of the mutant phenotype in the whole animal. Alternatively, one could employ virally mediated gene therapy by infecting mutants with a retrovirus containing PaK cDNA. Since many inborn errors of metabolism result from hepatic enzyme deficiencies, we expect that methods developed with these mutants will have implications even broader than those applying to PKU.

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