hph-1: A Mouse Mutant With Hereditary Hyperphenylalaninemia Induced by Ethylnitrosourea Mutagenesis

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ABSTRACT

Ethylnitrosourea mutagenesis of spermatogonial stem cells and a three-generation breeding scheme were used to screen for recessive mutations that cause defects in phenylalanine metabolism leading to elevated serum levels of this amino acid. This paper describes the isolation of such a mutation, *hph-1*, causing a heritable hyperphenylalaninemia in the neonate and weanling and an inability to effectively clear a phenylalanine challenge in the adult. Micro-pedigree analysis of the original mutant mouse and data obtained from crosses of affected and unaffected animals indicate that the mutation segregates in an autosomal recessive manner. An interspecies mouse backcross mapping experiment places the mutant gene locus on mouse chromosome 14 very near *Np-1* and a backcross experiment with a conventional inbred mouse strain involving a nearby locus confirms the chromosome 14 assignment. The initial symptomatology of the mutant phenotype suggests this mutant may represent a useful animal model for the study of hyperphenylalaninemia in man.

THE availability of mutants and the possibility of generating additional ones is an extremely useful, if not essential, element of any research program aimed at understanding particular biological pathways. The spectrum of mammalian mutants currently available is however relatively limited and such mutants are complex and poorly understood.

The report of an unusually efficient chemical mutagen of male mouse spermatogonial cells, *N*-ethyl-*N*-nitrosourea (ENU) (RUSSELL *et al.* 1979) led several investigators to initiate experiments designed at evaluating its potential for obtaining specific mutants needed for both basic and applied research. An initial set of experiments demonstrated that this approach was useful for inducing mutations throughout the mouse genome (JOHNSON and LEWIS 1981). Later experiments demonstrated the efficacy of ENU mutagenesis for producing mutants in specific areas of the mouse genome (*i.e.*, T/t region of mouse chromosome *17*) (BODE 1984; SHEDLOVSKY *et al.* 1986; JUSTICE and BODE 1986).

This paper further documents the practicality and power of ENU mutagenesis. It describes the induction, isolation and genetic characterization of a mutation causing hyperphenylalaninemia (HPA) in the mouse that may provide an animal model for the study of HPA in humans (for a general review of human HPA syndromes see TOURIAN and SIDBURY 1983). This recessive mutation has been designated hyperphenylalaninemia, symbol *hph* and, since several other genes are able to produce similar phenotypes due to the rather complex metabolic pathway involved, we have provisionally ranked this mutation *hph-1*.

The most common HPA syndrome in humans is referred to as classical phenylketonuria (PKU) and is a heritable metabolic disorder that results in a deficiency of the enzyme phenylalanine hydroxylase (PAH) (EC 1.14.16.1) (SCRIVER and CLOW 1980). In untreated patients, the clinical picture usually includes irreversible impairment of postnatal brain development and mental retardation of variable severity. The disease is transmitted as an autosomal recessive trait whose frequency varies considerably between populations. In the United States the allele frequency has been estimated at 0.01 (TOURIAN and SIDBURY 1983). Other HPA syndromes, though less frequently encountered in the human population, also are accompanied by neurological disorders.

Due to the absence of a genetically based animal model for HPA, considerable efforts have gone into developing chemically induced models (BERRY *et al.* 1975; DELVALLE, DIENEL and GREENGARD 1978; WAPNIR and MOAK 1979; COTTON 1986). Although helpful in some regards, the use of chemical inhibitors leads to some uncertainty in data interpretation due to possible secondary effects. We think that the mutation we report here may provide a useful model for studies in comparative pathology, therapeutics and disease prevention without these inherent uncertainties.

MATERIALS AND METHODS

Mouse strains: Mutagen-treated males were (C57BL/6 × CBA/Ca)F_1 animals and were crossed to females of the same genotype. While there are advantages for subsequent physiological and molecular studies accruing from inducing mutations in a pure inbred genetic background, we reluc-

tantly sacrificed these in this experiment to profit from the greater fecundity that mutagen-treated F_1 males exhibit compared to the component inbred strains. Such males also tolerate higher doses of ENU without being permanently sterilized or killed. In terms of DNA sequence analysis, however this means that the mutation reported here could have been induced in either C57BL/6 or CBA/Ca chromatin.

Mutagenesis: Stock ENU (purchased from Bioclinical Laboratories, Bohemia, New York) was assayed for activity by the colorimetric method of PACHOLEC and CHAPMAN (1985) (data not shown). Males were treated with mutagen by intraperitoneal injection of either a single dose of 250 mg ENU/kg body weight or with multiple injections of 100 mg/kg given at weekly intervals over a period of 3 or 4 weeks. Other details of the mutagenesis procedure are as reported in BODE (1984). Briefly summarizing, immediately prior to injection sufficient 0.1 M (pH 4.5) potassium phosphate buffer was added to ENU crystals to give a final concentration of 15 mg/ml. All operations were done in an efficient chemical hood and the injected animals were maintained in the hood for several days after treatment. Mutagen-treated males were caged with females 7–8 weeks after treatment and on regaining fertility were caged with new females on a weekly basis.

Screening: To identify animals with a higher than normal blood level of phenylalanine (PHE), we used the bacterial growth inhibition method of GUTHRIE and SUSI (1963). The amount of PHE in a dried blood sample is estimated from its ability to support the growth of *Bacillus subtilis* in minimal medium containing an inhibitor of PHE synthesis. [The intermediates of PHE metabolism, phenylpyruvate and phenyllactate, also permit bacterial growth (BIXBY, PALLATAO and PRYLES 1963) but their presence at high levels in blood would also suggest a mutation worthy of further study.] To perform a test, a drop of blood was collected from each animal on a piece of filter paper. A disc of suitable diameter was punched from this and incubated on agar containing minimal medium, the inhibitor and *B. subtilis* spores. The PHE concentration of the blood sample was estimated by comparing the bacterial growth ring around the test discs with those observed around control discs containing known amounts of PHE. We utilized a commercially available test kit (Sigma kit 160-B) allowing 48 samples to be analyzed on one 13 x 18 x 3 cm plastic dish. The plates were incubated for 16–18 hr at 37° prior to measuring growth zone diameters.

To photograph the assay plate the surface of the agar was flooded with a freshly prepared 0.5% (w/v) solution of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride (Sigma Chemical). After 5 min the solution was decanted, the surface rinsed with distilled water and a photograph taken. The agar gel is stained a dark purple but the bacterial growth rings remain unstained and are seen as clear halos. Because the clear halos diffuse outward with time, recorded growth measurements were those from the unstained plates.

Blood from normal mice gives growth rings corresponding to 2–4 mg/dl (0.12–0.24 mM) PHE. When blood from a member of a litter gave a growth ring larger than the 4 mg/dl control disc this identified the parents as putative carriers of an *hph* mutation and further litters from them were examined.

Linkage analysis: To map the *hph-1* gene we used an interspecific mouse backcross of the type (*Mus musculus domesticus* × *Mus spretus*) F_1 female × *M. musculus domesticus* male segregating for *hph-1*. Since the two species, *M. m. domesticus* on the one hand and *M. spretus* on the other, differ at many coat color, biochemical, and antigenic loci in addition to restriction enzyme cleavage sites it is possible to explore a large portion of the mouse chromosome map using a single backcross (BONHOMME, MARTIN and THALER 1978). Seventy offspring were typed for 44 segregating loci (BONHOMME *et al.* 1984) as well as for their serum PHE concentration (GUTHRIE and SUSI 1963) allowing their classification as either homozygotes or heterozygotes for the *M. m. domesticus* variant of the polymorphic loci and for the *hph-1* mutation. Also, blots of genomic restriction enzyme digests of interspecific backcross animals were hybridized with a rat PAH cDNA clone (DAHL and MERCER 1986) according to the method of SOUTHERN (1975) to determine the segregation of an *Eco*RI restriction fragment length polymorphism associated with the PAH structural gene.

A subsequent mapping study was done using a similar backcross to a conventional inbred mouse strain (*i.e.*, BUB/BnJ) carrying a polymorphic esterase-10 enzyme on chromosome *14* near the *Np-1* gene (WOMACK *et al.* 1977). *hph-1* homozygotes were mated to BUB/BnJ animals. The F_1 progeny were backcrossed to the *hph-1* homozygous parent and the backcross progeny were assessed with respect to their *hph-1* and *Es-10* phenotypes.

RESULTS

Carrier screening: It was unlikely *a priori* that the GUTHRIE screen would detect the usual heterozygote carrier of a mutation causing HPA, since, at least in humans, the levels of PHE in the blood of carriers are near normal. However, since PHE metabolism is complex it was considered possible that mutagenesis could induce rare "spoiler" type mutations acting in a dominant manner that could therefore be detected in the heterozygous state. Since little is known about the mouse enzymes or their subunit organization, and the assay is relatively easy to perform, we decided to screen the F_1 progeny of mutagenized males. We tested animals at 5–6 weeks of age when they are ready for discard after screening associated with other mutagenesis experiments. No positive tests were observed in the testing of 7015 first generation offspring. We conclude that, if *hph* mutants are produced at the average per locus frequency of 1 mutation in 1500 gametes seen at other genetic loci (BODE 1984), the test cannot readily allow carrier detection. We also conclude that when screening whole mouse blood for elevated PHE with the GUTHRIE assay, false positive readings are very rare in mice in this age group.

Generation of homozygous mutants: Having established that it was necessary to produce homozygous mutant animals, the three-generation breeding scheme outlined in Figure 1 was undertaken. A given ENU-mutagenized male was caged with wild-type females to produce approximately 25 male progeny, generation-1 (G-1). Each G-1 male was mated to a wild-type female to produce 2–4 G-2 daughters that were then mated back to their fathers (backcross). For any given mutant locus (m_q), half of the daughters

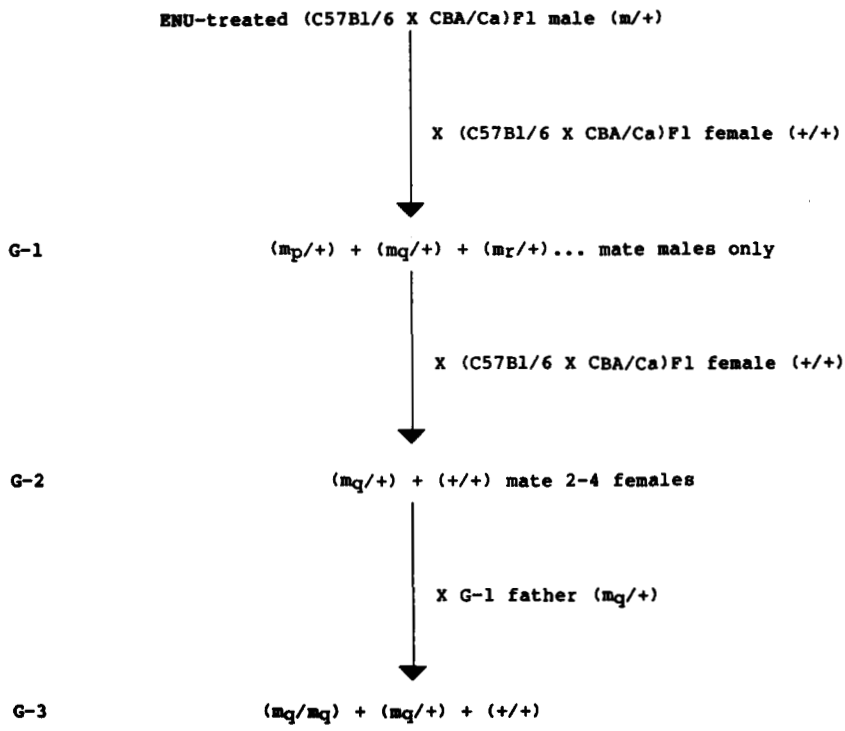

FIGURE 1.—Outline of the three-generation breeding scheme utilized to produce homozygous mutant animals.

will be mutant heterozygotes, like their fathers, and half will be wild type. If a daughter is heterozygous, one quarter of her G-3 progeny will be homozygous for mutation *q* and will express the particular recessive trait associated with a defect in gene *q*. The occurrence of such animals indicates that the G-1 male and the G-2 daughter are carriers for the mutation in *q*. Given that a particular G-1 male is heterozygous for a desired recessive mutation, that litters from three G-2 daughters are analyzed and that their average G-3 litter size is 5 there is a 77% probability that a G-1 male carrier for a recessive mutation will be detected by the outlined procedure. Because our previous studies (BODE 1984) indicated that an ENU-mutagenized male produces sperm from at least 100 independently mutagenized clones of spermatogonial cells, we analyzed about 25 sons (G-1 males) from each. A sample of this size from the sperm repertoire of a given male should include very few identical members. Although the three-generation breeding scheme depicted in Figure 1 is applicable to recessive mutations in general and thus the G-3 progeny could have been screened for a large number of different mutations, we chose to focus on those causing high blood levels of PHE. The G-2 litter from each G-1 male was culled to 2–4 daughters at weaning. These remained caged with their father (backcross) and their G-3 progeny were sacrificed and tested at 1 week of age. Since we expected most animals to be negative, this is a relatively efficient way of obtaining a single type of mutant. Only one cage is required per G-1 male tested. When the rare positive is encountered, other litters can be grown to weaning and tested. One quarter of the offspring from a carrier daughter will be homozygous mutant. Those daughters that are not carriers produce litters in which one-half of the animals are carriers, so the risk of identifying but then losing a mutant allele is low.

The average litter size remained very constant in G-1 and G-2 litters, with the former being 7.18 (±2.26) and the latter 7.02 (±1.82). The average G-3 litter size of 4.56 (±2.26) indicates that nearly three members of an average litter are never born, presumably due to homozygosity of newly-induced recessive embryonic lethal mutations. The most common visible mutant phenotypes seen in G-1 and G-2 animals are dominant cataract formation and dominant white-spotting. Because G-3 litters are sacrificed at 1 week of age, there is little opportunity to observe these animals for the development of visible phenotypes. Therefore the most notable feature with regard to G-3 litters is the reduction in average litter size mentioned above.

G-1 sons (105) from 19 mutagen-treated males were screened by the protocol described above. Among the 105 was one male that carried a recessive mutation causing HPA. This initial *hph-1* carrier animal was one of 26 male offspring that were tested from a particular mutagenized male. The positive GUTHRIE reading that identified the *hph-1* carrier male was the only abnormal reading (*i.e.*, outside the normal 2–4 mg PHE/dl whole blood range) seen in screening the progeny of the 105 G-1 males.

A typical assay plate with a positive sample is shown in Figure 2. When assays are performed 1 week after

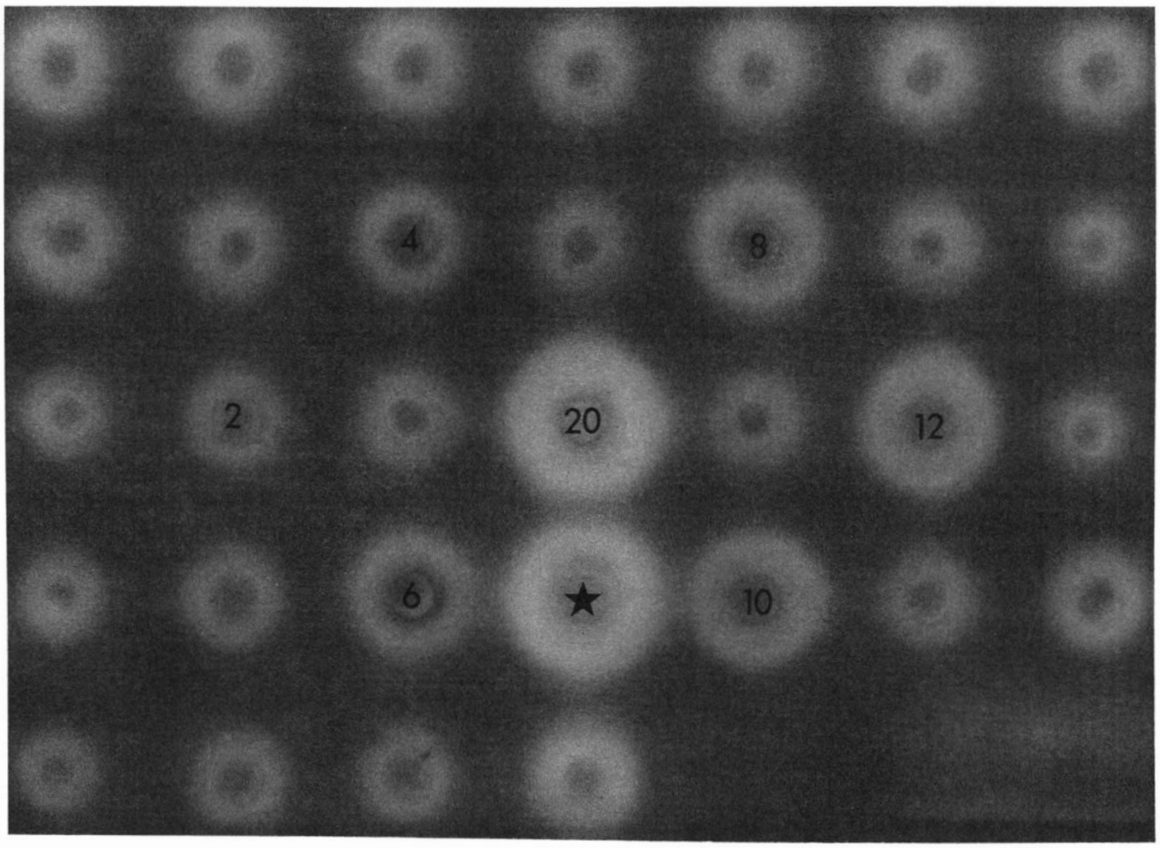

FIGURE 2.—Representative GUTHRIE assay plate used in screening for hyperphenylalaninemic mice. The numbered growth halos represent the results from control discs impregnated with whole blood containing the stated number of mg PHE per dl of blood. The (*) symbol indicates a growth halo of sufficient diameter to be considered a positive reading (*i.e.*, greater than the diameter of the growth halo from the 4 mg PHE/dl whole blood control disc).

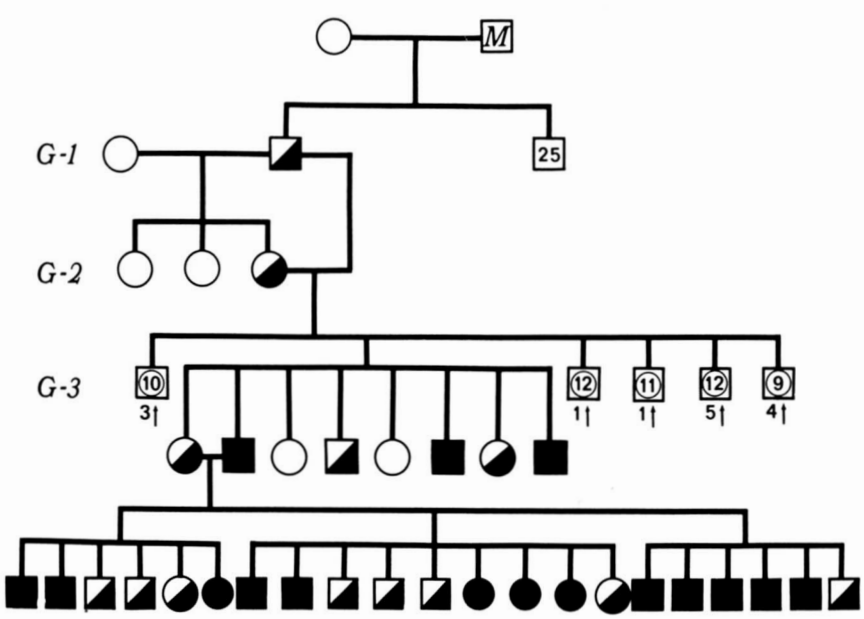

FIGURE 3.—A partial pedigree of the original ENU-treated male (symbolized by the letter M) depicting the induction, identification and transmission of the mutant allele *hph-1*. *Circles* represent females and *squares* males. The designated genotypes homozygous mutant (closed circles and squares), heterozygous mutant (*half-closed circles* and *squares*) and wild type (*open circles* and *squares*), were assigned on the basis of phenotype and breeding data in the same manner as described in the legend to Table 1. In some cases, for simplification purposes or where animals were not progeny tested, the total number of animals in a litter is depicted within a circle and a square with the number of affected animals beneath next to an *arrow* pointing up indicating that this is the number of animals that were found with elevated blood PHE levels. Three positive tests were observed in a litter of ten animals born to one of the G-2 daughters. Two of the daughters gave no hyperphenylalaninemic offspring when mated to their father (not shown in graphic) and were thus presumed to be noncarriers. The third, was a heterozygous mutant animal and 17 out of 62 (27.4%) of her offspring exhibited elevated blood PHE levels. One affected male from the second litter of the G-2 carrier female was mated to a carrier sister and 13 out of 21 (61.9%) the progeny were affected.

TABLE 1

Inheritance of hyperphenylalaninemic phenotype

Parental genotypes	Offspring phenotype		
	High PHE	Normal PHE	Total
1) *hph-1/hph-1* × *hph-1/hph-1*	71	0	71
2) *hph-1/hph-1* × *hph-1/+*	132(45.4%)	159(54.6%)	291
3) *hph-1/+* × *hph-1/+*	203(25.2%)	603(74.8%)	806
4) *hph-1/hph-1* × *+/+*	0	28	28

Animals that tested positive on the GUTHRIE assay gave all affected progeny when mated with similar animals and were thus inferred to be homozygous for the *hph-1* mutation (see cross 1). Animals that were negative when tested by the GUTHRIE assay but which gave positive progeny when mated to positive animals were inferred to be heterozygous for the *hph-1* mutation (see cross 2). Animals identified as heterozygous, when mated to other animals with the same characteristics gave positive offspring in a proportion consistent with autosomal recessive segregation (see cross 3). Animals that were negative when tested by the GUTHRIE assay and that gave no positive progeny when mated to positive animals were inferred to be wild type with respect to the *hph-1* locus (see cross 4).

TABLE 2

Mapping the *hph-1* locus relative to *Np-1*

Genotype	Frequency
Parental classes	
Np-1^a hph-1/Np-1^a hph-1	34
Np-1^a hph-1/Np-1^b +	24
Recombinant classes	
Np-1^a hph-1/Np-1^b hph-1	0
Np-1^a hph-1/Np-1^a +	0
Total examined	47
Total recombinant/total examined	0/47

TABLE 3

Mapping the *hph-1* locus relative to *Es-10*

Genotype	Frequency
Parental classes	
Es-10^a hph-1/Es-10^a hph-1	25
Es-10^a hph-1/Es-10^c +	33
Recombinant classes	
Es-10^a hph-1/Es-10^c hph-1	9
Es-10^a hph-1/Es-10^a +	5
Total examined	72
Total recombinant/Total examined = 14/72 = 0.19	

birth, the size of the growth zones indicates homozygotes have blood levels of PHE between 10 and 20 mg/dl (0.61 and 1.21 mM), compared to 2–4 mg/dl (0.12–0.24 mM) for both heterozygotes and normal mice. This was verified by quantitative spectrophotometric determination of serum PHE concentration by the method of SHEN and ABEL (1977) (MCDONALD and BODE 1987).

Segregation and symptomatology of *hph-1*. The inheritance of HPA is as expected for a single Mendelian gene acting in a recessive manner (see Figure 3 and Table 1). In preparing the partial pedigree represented in Figure 3, one homozygous male was misclassified at weaning. An analysis of its progeny strongly suggested that it was a homozygote even though the PHE level in its blood was normal when it was tested at 6 weeks of age. When this male was challenged with PHE in its drinking water (5 mg/ml) its blood level rose to 20 mg/dl (1.21 mM). This is the only instance of a false negative Guthrie assay result that we have observed. After this misclassification occurred we began to challenge all potential *hph-1* homozygotes older than 3 weeks of age. Prolonged exposure to PHE in the drinking water has frequently led to the death of homozygotes but not heterozygotes or wild type animals. Death occurs after a chronic wasting syndrome that is more prolonged the greater the age of the homozygous animal at the onset of PHE loading (data not shown). Unchallenged homozygotes are normally healthy and fertile in both sexes.

Linkage of *hph-1*. The chromosomal location of *hph-1* was initially determined by interspecies backcross. A total of 44 loci (including 15 of the 19 mouse autosomes, the *X* chromosome and some polymorphic loci not yet mapped to a particular chromosome) were examined for cosegregation with *hph-1*. *hph-1* was found to be closely linked to the *Np-1* (Nucleoside phosphorylase-1) locus on mouse chromosome *14*. The *M. spretus* mouse species (SPE/Pas) that we used carries a rare electrophoretical variant *Np-1^b* (BONHOMME *et al.* 1984) that neither C57BL/6 nor CBA/Ca strains carry. In all instances we found cosegregation of *hph-1* with the C57BL/6 and CBA/Ca *Np-1* allele (0 recombinants out of 47 backcross progeny examined) (see Table 2). This establishes the distance of *hph-1* from *Np-1* as less than 2 (± less than 2.0) cM. Later the *hph-1* locus was mapped relative to the *Es-10* (esterase-10) locus on mouse chromosome *14* using a backcross to a conventional inbred mouse strain (see Table 3). The mouse strain BUB/BnJ used for this backcross carries an esterase-10 enzyme (*Es-10^c*) that migrates slower than either the C57BL/6 or CBA/Ca enzyme (*Es-10^a*) during electrophoresis (WOMACK *et al.* 1977). These results show clearly that *hph-1* is also linked to *Es-10*; the two loci are separated by 19 (±4.6) cM. WOMACK *et al.* (1977) reported a recombination distance between *Es-10* and *Np-1* of 10.2 (±1.9) cM. While this suggests a gene order of *Es-10-Np-1-hph-1* we are reluctant to propose this due to the possibility of error in recombination interval calculation caused by heterogeneity in recombination between crosses involving conventional strains of mice and interspecific crosses involving *M. spretus* as reported by NADEAU *et al.* (1986).

TABLE 4

Mapping the *Pah* locus relative to c-*myb* locus

Genotype	Frequency
Parental classes	
Pah^m c-myb^m/Pah^mc-myb^m	20
Pah^m c-myb^m/Pah^sc-myb^s	19
Recombinant classes	
Pah^m c-myb^m/Pah^mc-myb^s	8
Pah^m c-myb^m/Pah^sc-myb^m	10
Total examined	57
Total recombinant/total examined	18/57 = 0.32

Linkage of the PAH structural gene: The *Eco*RI restriction fragment length polymorphism between *M. m. domesticus* and *M. spretus* at the phenylalanine hydroxylase structural gene locus (provisionally called *Pah*) allowed for the determination of possible cosegregation of this locus with other polymorphic loci in the same interspecific mouse backcross as described in the mapping of the *hph-1* locus. The *M. m. domesticus* allele is referred to as Pah^m and the *M. spretus* allele as Pah^s. Of all the polymorphic loci examined, only one showed detectable cosegregation with *Pah* (see Table 4). *Pah* cosegregated with the c-*myb* oncogene on mouse chromosome *10* in *39* out of 57 backcross progeny for a recombination interval of 32 (±6.2) cM.

DISCUSSION

The rate-limiting step in the catabolism of phenylalanine is catalyzed by the enzyme PAH and a deficiency of this hepatic enzyme is the most common cause of HPA in humans (SCRIVER and CLOW 1980). The reaction catalyzed by PAH requires the cofactor 5,6,7,8-tetrahydrobiopterin that is maintained in the appropriate state of reduction by the enzyme quinonoid dihydropterin reductase (EC 1.6.99.7) (KAUFMAN 1976; AMAREGO, RANDLES and WARING 1984). Defects in the biosynthesis of tetrahydrobiopterin or in the activity of the reductase can also lead to the hyperphenylalaninemic state. In terms of mutagenesis, one can thus expect at least three direct ways to generate a hyperphenylalaninemic mouse: (1) mutations reducing PAH apoenzyme activity, (2) mutations interrupting cofactor synthesis and (3) mutations reducing the reductase enzyme activity. The biochemical nature of the *hph-1* mutation will require more investigation prior to its classification into one of these general areas.

Although the *hph-1* mutant requires additional study in order to evaluate its specific use as an animal model for PKU, its isolation provides a paradigm for obtaining mice having other metabolic defects. The mouse is an excellent subject for minor surgery and (in addition to blood) a single kidney or testis or a liver biopsy can easily be removed for biochemical or immunological studies with minimal risk of compromising the subsequent fertility or viability of the animal. Mutant isolation could be carried out as an ongoing activity in a modest-sized animal colony. Although it is more efficient to screen G-3 progeny for a larger number of traits, there are advantages in undertaking a highly focused search such as the one reported here. In addition to the inherent value of the mutant and the information that may be obtained from it, we hope that this study will alert mammalian biologists working in a variety of fields to the availability of this method for obtaining mutants, and will stimulate consideration of which specific mouse mutant phenotypes would be informative in particular systems, and lead to the design of new ways for detecting and using such mutants.

The enzyme PAH has been studied more extensively in the rat than in the mouse but there are many reasons to believe that the mouse system is similar to the rat. Hybridization of mouse genomic DNA with a rat PAH cDNA probe suggests a high degree of sequence similarity between the mouse and rat gene. Thus, we were able to use a cDNA probe of the rat PAH gene to probe restriction enzyme digests of genomic DNA from interspecific backcross offspring. On the basis of the segregation of an *Eco*RI restriction site polymorphism discovered between *M. m. domesticus* and *M. spretus*, we found no evidence of linkage between the PAH structural gene and *Np-1*. In humans, PAH is encoded by a single gene. This has been shown by reconstituting PAH enzyme activity from a cDNA molecule (LEDLEY *et al.* 1986) and by cloning a unique chromosomal locus for this cDNA (DILLELA *et al.* 1986). The allelic variations of PAH in rats (MERCER *et al.* 1984) suggests the same explanation for rats. While there is no direct evidence of a single PAH locus in the mouse this is the logical inference. Thus, the HPA that we are considering in *hph-1* mutant mice is probably not the direct consequence of a mutation within the structural gene of PAH. The PAH gene itself exhibits some linkage with the c-*myb* oncogene on mouse chromosome 10, an observation consistent with a similar recent finding by LEDLEY *et al.* (1987).

PKU in humans results from mutations in the structural gene for PAH (LEDLEY, DILELLA and WOO 1985). Thus the *hph-1* mutation is not analogous to PKU in humans. However, the ease with which blood PHE levels can be manipulated in this mutant and the pathology that ensues should provide an interesting model for the study of chronic HPA in a mammalian system. In addition, because the etiology of the *hph-1* mutation appears to stem from an

abberration of biopterin metabolism (McDONALD and BODE 1987), this mutation should prove valuable in characterizing more atypical forms of HPA.

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