A Genetic Outlook on Phenylketonuria

Introduction

In 1934 Ivar Asbjrn Følling, a Norwegian scientist, noticed that a group of mentally retarded patients had a peculiar odor. It was determined that the odor was phenylacetic acid and that these individuals had large amounts of phenylpyruvic acid in their urine. Thus the name Phenylketoneuria was born.

Within 20 years of its discovery the management of PKU was understood. Through a selective diet and a protein supplement, first developed by Horst Bickel, people with PKU could lead mostly normal lives.

The Guthrie Test, invented in 1958, was the first wide spread screening for PKU. Newborn screening tests were done on every newborn from 1960 on.

Phenylketoneuria is a autosomal recessive disorder in amino acid metabolism. A deficiency in the hepatic enzyme phenylalanine hydroxylase (PAH) causes the individual to be unable to convert phenylalanine to tyrosine. This results in an excess of indigestible phenylalanine in the blood and eventually the brain which causes mental retardation and various other neurological problems.

PAH is a 100 kb region of DNA on chromosome 12, band region q22-24.1. The gene contains 13 exons and a complex array of 5' cis-acting factors, trans-activated regulatory elements. There are more than 500 mutations in this gene that can lead to PKU.

Phenylalanine hydroxylase gene (OMIM)

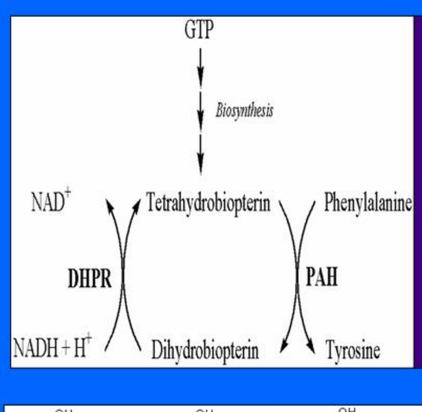
Biochemistry

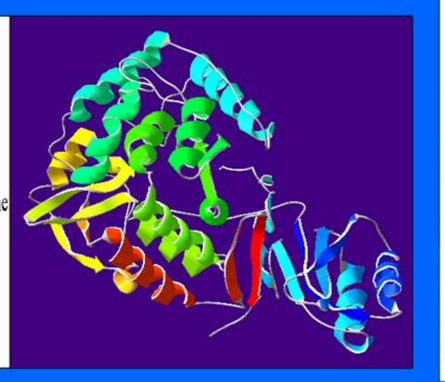
Classical PKU results from a defective gene coding for enzyme phenylalanine hydroxylase (PAH). PAH is an enzyme involved in amino acid metabolism, normally converting phenylalanine to tyrosine. Essentially PKU results from the body's inability to metabolize phenylalanine. If PAH is defective, the levels of phenylalanine in the body are far greater than normal, while levels of tyrosine are far below.

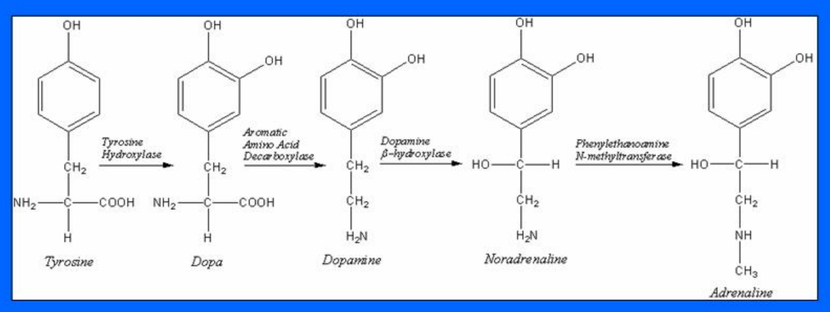
Phenylalanine hydroxylase (PAH) is a tetramer composed of two dimmers coded for by the *PAH* gene. Each subunit is in turn composed of three domains, a regulatory domain, a catalytic domain, and a tetramerization domain. Functions when in the presence of cofactor Tetrahydrobiopterin or BH_4 . Homologous to two other enzymes; tyrosine and tryptophan hydroxylase. The enzyme adds a hydroxyl group to the 6 carbon ring of phenylalanine, converting it to tyrosine. Intracellular concentrations of BH_4 are controlled by the enzyme GTP cyclohydrolase I (GTP-CH). Since the activity of PAH requires BH_4 and the BH_4 level is determined by GTC-CH then PAH is controlled by GTC-CH.

Phenylalanine is an essential non-polar amino acid with a benzene ring obtained through diet. In nature it exists in the L conformation. Phenylalanine is a large neutral amino acid (LNNA) able to cross the blood-brain barrier via large neutral amino acid transporters (LNNAT). Increased levels of phenylalanine in the blood result in the saturation of LNNATs along the blood-brain barrier. Such saturation decreases the body's ability to transport phenylalanine and other LNNAs such as tryptophan into the central nervous system. Adequate transport levels of certain amino acids across the BBB are essential in the synthesis of a host of neurotransmitters which require them as catabolic precursors.

Tyrosine is another essential amino acid involved in a host of cellular functions including cell signaling, protein synthesis, and a precursor for the synthesis of certain neurotransmitters including dopamine, epinephrine, norepinephrine and serotonin.







Improving Identification

PKU is traditionally identified in newborns through the Gurthrie test, but during the 1990's efforts were made to develop prenatal tests for families known to be at risk for PKU using an array of molecular genetics tools, including linkage analysis and genetic sequencing.

Treatment Today

Conventional treatment for PKU is a controlled diet, but the diet is extremely restrictive and patients must remain on it indefinitely. Several alternative avenues of treatment are under active exploration. One proposed therapy makes use of a common large neutral amino acid (LNAA) transporter to lower phenylalanine concentrations in the brain [Matalon 2003]. Other research suggests that phenylalanine ammonia lyase (PAL) may be administered as a palliative in conjunction with a modified diet. Phenylalanine ammonia lyase converts phenylalanine to ammonia and transcinnamic acid. Both are removed from the body or quickly converted to harmless metabolites [Levy 1999]. However, neither of these solutions addresses the fundamental genetic nature of phenylketonuria.

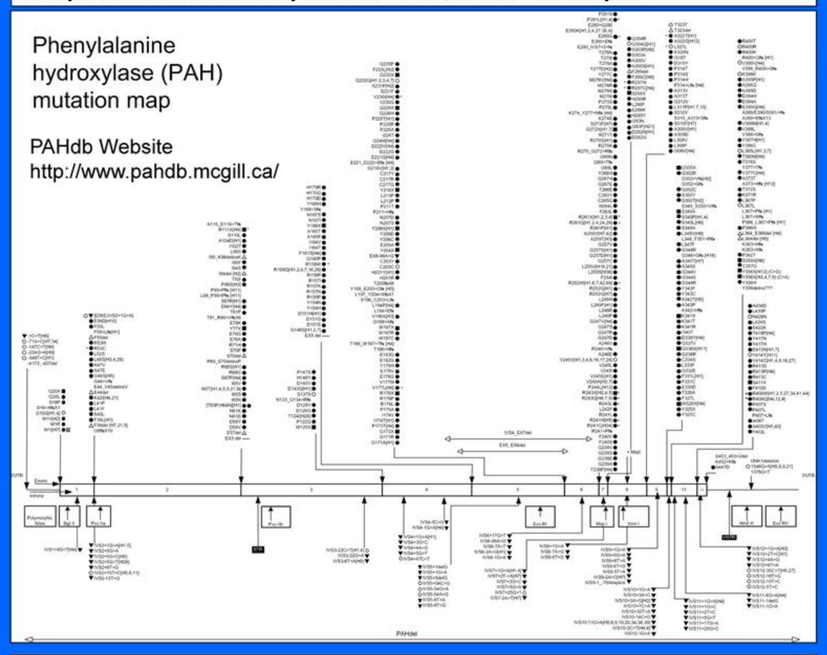
In the Works: Gene Therapy

Several research groups are exploring the possibilities of gene therapy. The greatest block to successful gene therapy of PKU is the development of a vector that is accepted by liver cells, efficiently produces the normal, wild-type enzyme, and persists in the modified cells. Several vectors have been investigated, but with limited success. One 2003 review found short-term prospects for PKU gene therapy discouraging. The review looked at several recent experiments and found that none of them reduced phenylalanine levels to normal levels on a permanent or long-term basis. The review also questioned the PAH^{enu2} mouse model most commonly used to study PKU, suggesting that differences between the mouse and human exon 7 might make the mouse an insufficiently rigorous model for some homology studies. Exon 7 codes a critical domain involved with binding tetrahydrobiopterin (BH4), an enzyme cofactor. [Zhaohing 2003]

Other groups have investigated treatments which do not require transformation of liver cells. Normally, PAH is exclusively expressed in the liver, but the metabolic effects are a result of high phenylalanine levels in the blood and brain. Researchers hope that stimulating PAH activity in other, more accessible tissues will lower blood phenylalanine levels in the same way that normal PAH activity in the liver does.

Genetic alteration of PKU locus

Many mutations have been identified at the PAH locus: missense, nonsense, tandem repeats, etc, all may contribute to a functionally identical – that is, defunct - PAH enzyme.



Known mutations at the q22-q24.1 PAH locus on chromosome 12. Introns are represented by the numbered white boxes; exons are the black lines separating boxes. Mutations that affect mRNA splicing are shown below the gene diagram; all others are shown above it. The plethora of known mutations in exons 5-12 reflects the importance of those domains to correct protein function. Mutation map from the PAHdb website.

Prenatal Testing

Previously the only way to screen for PKU was after birth however this still has problems because the unborn child is effected by it and can acquire defects while in the womb. A prenatal diagnosis method is currently under investigation. One paper out of India describes a method for prenatal testing using restriction fragment length polymorphisms (RFLPs) and variable number of tandem repeats (VNTR; 30 bp long cassette in the 3' UTR). A family with one child diagnosed with PKU undergoes testing to determine if a second child also will suffer from the disorder. The father, mother, effected child (proband), and chorionic villus (placenta cells) sample were tested for RFLP and VNTR.

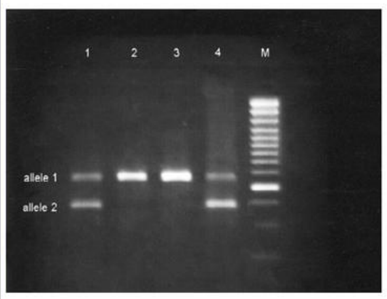


Fig. 1. Ethidium bromide stained 3 per cent agarose gel showing variable number of tandem repeats (VNTR) alleles. Lane 1: mother; Lane 2: father; Lane 3: proband; Lane 4: foetus; Lane M: 500 bp DNA ladder.

Fig. 2. Ethidium bromide stained 3 per cent agarose gel showing *Xmn*1 restricted fragment length polymorphism (RFLP) alleles. Lane M: qX 174 Hae III digest DNA ladder; Lane U: Undigest; Lane 1: mother (+/+); Lane 2: father (-/+); Lane 3: proband (+/+); Lane 4: foetus (+/+).

Linkage analysis of polymorphic markers enables prenatal diagnosis. The two gels above show the migration of the VNTRs and STRs for the PAH locus. The mother has two alleles in the VNTR marker gel. The father is homozygous for this allele and so is uninformative. It can be seen that the daughter inherited allele 1 from the mother and this must then be the disease gene. The foetus inherited allele 2 from the mother and thus it can be determined this is not the disease allele. For the RFLP analysis it can be determined that the father now has two alleles (+, -). The father gave the minus allele to his effected daughter and also to the foetus. The mother is homozygous at this locus and is therefore uninformative. Due to RFLP and VNTR analysis it can be determined that both mother and father are heterozygous at this locus. Both parents have a functional and a nonfunctional allele at this locus. The effected daughter inherited the two defective alleles. The foetus inherited one wild type copy from the mother and one mutant copy from the father and will not have PKU.

Gene Therapy: Bone Marrow

In one attempt to study the efficacy of indirectly providing PAH to the bloodstream, researchers created a strain of mice that expressed PAH in bone marrow, but not in the liver. Harding, Neff, *et al* transformed mouse embryos so that a copy of the PAH enzyme was under control of a beta globin promoter expressed in bone marrow, and crossed this strain with *PAHenu2* mice. The offspring were monitored to see if the bone marrow PAH affected phenylalanine in the blood. However, the expressed PAH did not significantly impact phenylalanine blood concentrations, even after tetrahydrobiopterin (BH4) supplements were introduced to the mouse blood stream. BH4 is a PAH cofactor, and is normally not found outside the liver. Researchers speculated on several possibilities for the low impact of the expressed enzyme, but have not yet found a way to boost its efficiency. [Harding 2003]

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Gene Therapy: Retrovirus Mediated Gene Transfer in Skin Cells

A paper out of Denmark and Pennsylvania have devised an experimental procedure in which the skin is used as a metabolite sink. They performed their experiments with kerinocyte cell which make up about 90% of epidermis. There system involved utilizing an adenovirus to introduce to transgenes to the kerinocytes. Keratinocytes were chosen because of there close contact with the blood and that they already produce and secrete many proteins and have a high metabolic rate.

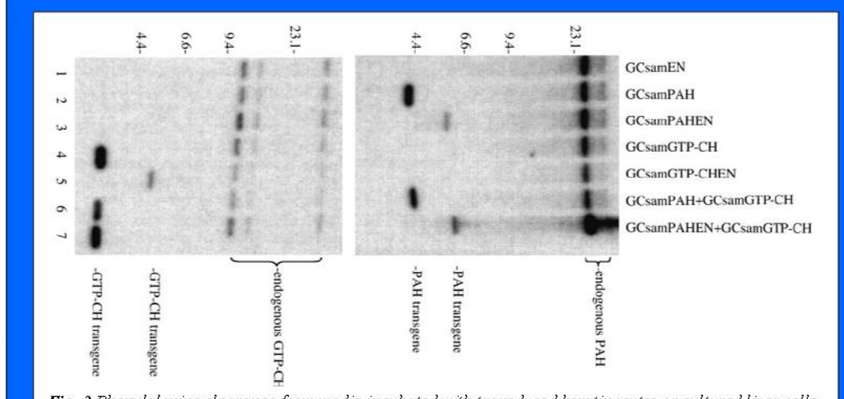


Fig. 3 *Phenylalanine clearance from media incubated with transduced keratinocytes or cultured liver cells (HepG2). Complete keratinocyte medium was incubated at 37°C with transduced keratinocytes or HepG2 cells grown to confluency in six-well plates. After 24 h the medium was removed from the cells and the phenylalanine concentration in the medium samples was determined. Data are given as the mean of triplicate determinations with the vertical bars. 'GCsamPAH + GCsamGTP-CH 1:1' indicates that cells consist of 1:1 mixtures of keratinocytes transduced with GCsamPAH and keratinocytes transduced with GCsamGTP-CH.*

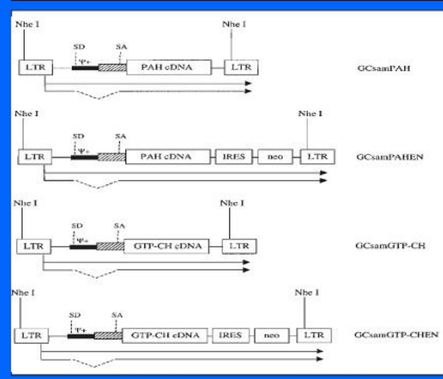


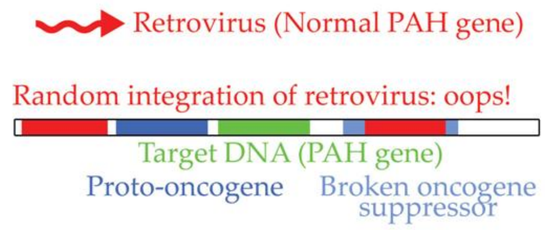
Fig. 4. Structures of the retroviral vectors used in this study. IRES, internal ribosome entry site from encephalomyocarditis virus; SD, splice donor site; SA, splice acceptor site; C+, retroviral packaging signal; neo, neomycin phosphotransferase II gene. The restriction sites for NheI are indicated. Vectors are not drawn to scale.

Discussion

In this experiment transgenes containing both GTP-CH and PAH were introduced to a keratinocyte cells. It shows that in the cells that had uptake of both GTP-CH and PAH should significant clearance of phenylalanine. In cells where only one of the transgenes was transposed phenylalanine was not significantly cleared. These figures show the dramatic increase in PAH and GTP-CH production from the endogenous level. This experiment proposes a possibility in humans to genetically alter the skin cells in order to cure PKU. PKU is deemed as a useful model for this system in that it can get around the targeted destruction by the immune system. In many such systems the transgene will not have a lasting effect due to the immune system destruction of the product. It is hypothesized that the PKU system may be different in that most PKU sufferers still produce a protein it is just inactive. The native protein may resemble the transgene protein enough to allow the protein to survive targeting by the immune system.

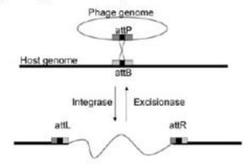
Gene Therapy: Liver Studies

Retroviruses integrate their own DNA into the genome of host cells, opening the possibility of directly correcting phenylketonuriacs' mutated DNA. However, investigation into retroviral vectors is losing popularity as the possibilities of retroviral oncogene activity are more fully realized [Ding 2004].

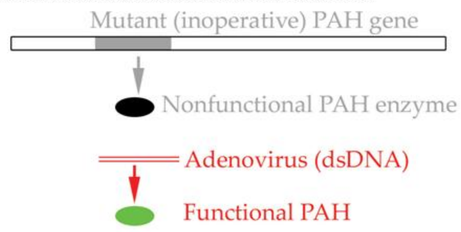


More recent studies suggest that some of the problems seen in earlier experiments may have been partially resolved. In the last year, two experiments, one using an adeno-associated virus (AAV), and one using a bacteriophage, have effected persistent reductions in PAH levels in mice.

Adenoviruses consist of double-stranded DNA surrounded by a protein coat. Adenovirus DNA does not integrate into host DNA, but uses the cell's machinery to replicate itself. Early experiments using adenoviruses as a PKU therapy vector suffered from problems with persistence in mouse experiments [Ding 2004]. The mouse immune system recognized the vector as a pathogen, removing the vector and the corrected PAH gene it carried. One group claims to have overcome this problem using adeno-associated virus (AAV), but this does so by suppressing the immune system [Oh 2004]. Earlier studies suggest that adeno-associated viruses might preferentially integrate into quiescent, not active, cells, defeating the purpose of gene therapy.



One promising study used a bacteriophage vector *in vivo* to deliver a wild-type PAH transcript to mouse liver cells. The bacteriophage carried a site-specific integrate which catalyzed recombination at the PAH locus. Test mice showed persistent declines in phenylalanine levels. [Chen 2005]



Future Areas of Research

Researchers have successfully completed a liver-directed gene transfer of the mouse PAH gene into hyperphenylalaninemic PAH-deficient Pah(enu2) mice, the equivalent of a human with PKU. The gene was transferred via a recombinant adeno-associated virus vector. The transfer was associated with significant increases in phenylalanine clearance and was able to correct phenylalanine levels in the blood. This experiment has promising possibilities for PKU treatment in the future, however questions about its long term safety and expression stability remain.

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