retained significant ability to competitively inhibit invasion. They had less binding affinity than the intact molecule, however, and the smaller structures were the least effective in inhibiting invasion. These observations suggest that the lactosamine chains of erythroglycan may be the primary interaction binding site, but that the core, after endo-β-galactosidase treatment, retains enough lactosamine structures to be active. Indeed, erythrocytes treated with endo-β-galactosidase are still susceptible to infection.

The ability of band 3 eythroglycan to strongly inhibit invasion implicates this structure as an important parasite-binding site and supports a role for band 3 in mediating the transmembrane modulation of the erythrocyte cytoskeleton during invasion. It has been observed that chymotrypsin cleaves band 3 between the erythroglycan attachment site and the cytoplasmic domain but has no effect on infectibility (*20*); however, neither of the cleaved fragments is released from the cell membrane and both are still strongly associated even after solubilization of the membrane with nonionic detergent (*21*). Thus chymotrypsin cleavage of band 3 would not interfere with communication between erythroglycan and the cytoplasmic domain.

In malaria the merozoite binds to the erythrocyte surface and disrupts the forces that maintain the shape of the cell and that ordinarily prevent the entry of foreign bodies. The lactosamine oligomers of band 3 erythroglycan appear to mediate part of this process. We showed earlier that α_1-acid glycoprotein blocks merozoite attachment as part of the natural protective response (*1*). Similarly, synthetic chemotherapeutic agents resembling polylactosamine may prove effective against this disease.

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Gene Transfer and Expression of Human Phenylalanine Hydroxylase

Abstract. *Phenylketonuria (PKU) is caused by a genetic deficiency of the enzyme phenylalanine hydroxylase (PAH). A full-length complementary DNA clone of human PAH was inserted into a eukaryotic expression vector and transferred into mouse NIH3T3 cells which do not normally express PAH. The transformed mouse cells expressed PAH messenger RNA, immunoreactive protein, and enzymatic activity that are characteristic of the normal human liver products, demonstrating that a single gene contains all of the necessary genetic information to code for functional PAH. These results support the use of the human PAH probe in prenatal diagnosis and detection of carriers, to provide new opportunities for the biochemical characterization of normal and mutant enzymes, and in the investigation of alternative genetic therapies for PKU.*

Phenylketonuria, the most common inborn error of amino acid metabolism, is caused by a deficiency of the hepatic enzyme phenylalanine hydroxylase (PAH) (*1*). Most of the biochemical characterization of PAH has been done with enzyme purified from rat liver, which contains 20 to 30 times more PAH activity than the human liver. The purified rat enzyme has an apparent molecular size of 100 kilodaltons (kD) and consists of two subunits of approximately 50 kD each (*2, 3*). The human enzyme has been purified and also appears to be a dimer of 50 kD subunits (*4, 5*). There has been considerable controversy concerning the identity of the monomeric subunits. Several investigators have reported finding multiple forms of the enzyme which differ in size, charge, activity, and antigenicity, and two distinct bands representing the monomeric protein can be separated

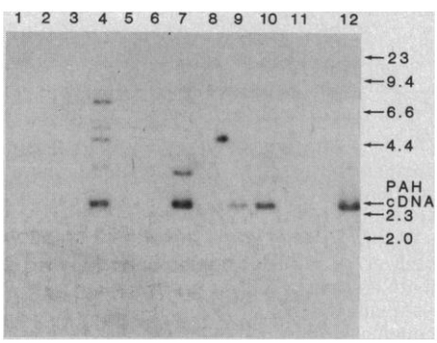

Fig. 1. Southern blot analysis of NIH3T3 cells transformed with MPAH(+) and pSV2NEO. (Lanes 1 to 10) DNA from transfected MPAH(+) and SV2NEO cell lines MPN1 to MPN10 (10 μg); (lane 11) DNA from cells transfected with SV2NEO alone (10 μg); (lane 12) MPAH(+) plasmid DNA (20 pg). DNA was digested with Eco RI, run on a 0.8 percent agarose gel, transferred to nitrocellulose paper, and hybridized with the purified Eco RI insert from phPAH247 (*8*). Filters were washed in 2× SSC (standard saline citrate) and 0.1 percent sodium dodecyl sulfate (SDS) for 1 hour at room temperature and 1 hour at 68°C, and then in 0.2× SSC and 0.1 percent SDS for 1 hour at 68°C.

on sodium dodecyl sulfate (SDS)–polyacrylamide gels (*2*, *6*). These findings suggest that PAH may be a heterodimer of different subunits. Other data, however, indicate that the rat enzyme is a homodimer of a single subunit which may undergo posttranslational modifications (*7*). In order to understand the genetics of PKU it is necessary to determine whether the subunits of the human enzyme are encoded by a single gene or by two distinct genes.

Rat and human PAH complementary DNA (cDNA) clones (*6*, *8*) and a full-length human PAH cDNA clone (phPAH247) comprising 2448 bases (*9*) have been isolated. Clone phPAH247 contains a 5′ untranslated sequence of 222 bases, an open reading frame of 1356 bases, and a 3′ untranslated sequence of 870 bases including a polyadenylation signal and a 19-base polyadenylate tail. The predicted amino acid sequence would result in a protein of 451 amino acids with a molecular size of 51.9 kD (*2*–*6*). This sequence and the predicted amino acid composition are consistent with data derived from the rat enzyme (*9*, *10*).

To determine whether this full length cDNA clone contains all of the genetic information necessary for expression of PAH enzymatic activity, the 2.4-kilobase (kb) cDNA insert from phPAH247 was subcloned into an expression vector containing the promoter and the cap-site of the human metallothionein gene (MT-II). The MT-II promoter has been used to express heterologous genes in gene transfer experiments (*11*). The recombinant clone, designated MPAH(+), was cotransfected with pSV2NEO into the mouse cell line NIH3T3 by means of the calcium phosphate precipitation method (*12*). Ten genticin-resistant colonies (*13*) were subcloned and designated MPN1 to MPN10. DNA from these colonies was examined for the presence of stably inte-

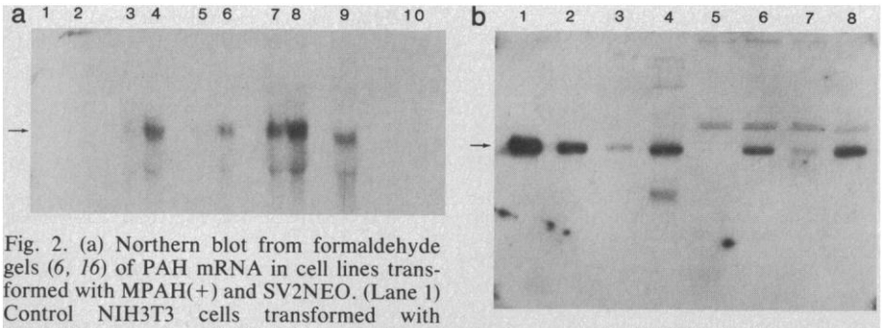

Fig. 2. (a) Northern blot from formaldehyde gels (*6*, *16*) of PAH mRNA in cell lines transformed with MPAH(+) and SV2NEO. (Lane 1) Control NIH3T3 cells transformed with SV2NEO alone; (lane 2) SV2NEO-transformed cells exposed to 5 μM $CdCl_2$ for 12 hours prior to harvest; (lanes 3, 5, and 7) cell lines [transformed with MPAH(+) and SV2NEO] MPN4, MPN9, and MPN10, respectively, without $CdCl_2$ induction; (lanes 4, 6, and 8) cell lines MPN4, MPN9, and MPN10 induced with 5 μM $CdCl_2$ for 12 hours prior to harvest; (lane 9) human hepatoma cell line hepG-2; (lane 10) 5 μg of poly(A)$^+$ RNA from mouse liver. Lanes 1 to 9 each contained 20 μg total RNA. Hybridization and wash conditions were as in Fig. 1. Arrow shows the position of PAH DNA (2.5 kb). (b) Western blot for PAH protein in cells transformed with MPAH(+) and SV2NEO after $CdCl_2$ induction. (Lanes 1, 2, and 3) rat liver extract (10 μg, 5 μg, and 1 μg); (lane 4) human liver (200 μg); (lane 5) SV2NEO-transformed NIH3T3 (200 μg); (lanes 6, 7, and 8) MPN4, MPN9, and MPN10, respectively (200 μg). Western blotting was performed with a 1:2500 dilution of affinity-purified goat antibody to rat PAH (*6*) and ^{125}I-labeled swine antibody to goat immunoglobulin G (10^8 cpm per microgram of protein) as described (*17*). Arrow shows position of PAH (51.9 kD).

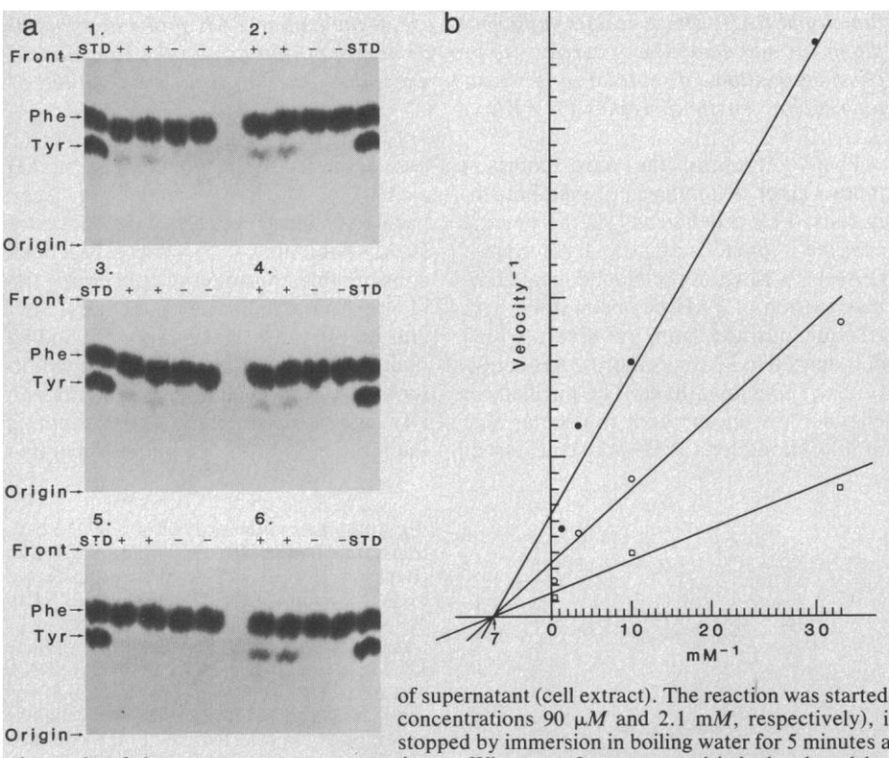

Fig. 3. (a) PAH activity in cells transformed with MPAH(+) and SV2NEO, and controls. The assay measures the production of [^{14}C]tyrosine from [^{14}C]phenylalanine in the presence of a crude cellular extract and the synthetic cofactor 6-methyltetrahydropterin (6MP) (*14*). The separation of [^{14}C]tyrosine from [^{14}C]phenylalanine by paper chromatography for six different cellular extracts is shown. Activity from the cell line MPN4 assayed without induction (panel 1) and after induction with $CdCl_2$ (panel 2). Activity from cell line MPN10 without induction (panel 3) and after induction with $CdCl_2$ (panel 4). (Panel 5) Activity in a control cell line transformed with PSV2NEO alone. (Panel 6) Activity in the human hepatoma cell line hepG-2. Assays were performed in duplicate in the presence (+) and the absence (−) of the pterin cofactor. The standard (STD) contained [^{14}C]phenylalanine and [^{14}C]tyrosine. Extracts were prepared from cells by three sonications (5 seconds each) at the lowest speed in a Branson Sonifier 185 and centrifugation for 5 minutes at 12,000*g*. The assay was done in a 100 μl volume containing 0.2M KPO_4 (*p*H 6.8), 0.8 mM phenylalanine, 400,000 cpm (0.01 mM) uniformly labeled [^{14}C]phenylalanine (500 mCi/mmol), 6 μg catalase, and 500 μg of supernatant (cell extract). The reaction was started with the addition of 6MP and dithiothreitol (final concentrations 90 μM and 2.1 mM, respectively), incubated for 1 hour at room temperature, and stopped by immersion in boiling water for 5 minutes and centrifugation at 13,000*g* for 5 minutes. A 25-μl sample of the supernatant was spotted onto Whattman 3mm paper, dried, developed by ascending chromatography in a solution of *n*-butanol:acetic acid:water (12:3:5), dried, and visualized by autoradiography. In this system, phenylalanine has an R_F of 0.75 while tyrosine has an R_F of 0.45. (b) Double reciprocal plot of activity and phenylalanine concentration in extracts of noninduced MPN10 (○), $CdCl_2$-induced MPN10 (□), and hepG-2 (●). PAH activity was quantitated after autoradiography by liquid scintillation counting of the region of chromatography paper (in Aquasol, New England Nuclear) corresponding to tyrosine. Values are given as the number of moles of tyrosine formed over the sample containing no cofactor. Linear regression was performed with the Advanced Statistical Analysis Package for the TRS-80 Model-III.

grated human PAH cDNA by Southern blot hybridization (Fig. 1). Hybridization and washing conditions were sufficiently stringent to allow the labeled probe to anneal to the human PAH gene, but not to the mouse chromosomal PAH gene. Four colonies contained the 2.4-kb Eco RI fragment (Fig. 1, lanes 4, 7, 9, and 10), corresponding to the human PAH cDNA from MPAH(+) (Fig. 1, lane 12).

RNA hybridization by Northern blot was done to determine if the heterologous human PAH gene was expressed as messenger RNA (mRNA) in these transformed cells (Fig. 2a). Control cells transfected with pSV2NEO alone did not have PAH mRNA (Fig. 2a, lanes 1 and 2). Each of the cell lines containing the intact PAH cDNA expressed a hybridizing mRNA approximately 2.5 kb in length (Fig. 2a, lanes 3, 5, and 7), consistent with the transcript expected from MPAH(+). Two experiments confirmed that this hybridizing mRNA was derived from the recombinant PAH clone and not from the endogenous mouse PAH gene. First, no hybridization was observed between the labeled human PAH probe and mouse liver mRNA under the conditions used in this experiment (Fig. 2a, lane 10). Second, transcription of the hybridizable PAH mRNA was induced by $CdCl_2$ (Fig. 2a, lanes 4, 6, and 8); this induction is characteristic of genes transcribed from the metallothionein gene promoter (*11*).

In order to demonstrate that the cell lines transformed with MPAH(+) and SV2NEO contained immunoreactive PAH, Western blotting with a PAH-specific antibody was performed (Fig. 2b). A distinct band of cross-reactive material which comigrated with rat PAH (Fig. 2b, lanes 1 to 3) and human PAH (Fig. 2b, lane 4) was present in cells transformed with MPAH(+) and SV2NEO (Fig. 2b, lanes 6 to 8) but not in SV2NEO-transformed cells (Fig. 2b, lane 5). The parent NIH3T3 cells contained another cross-reactive band of about 60 kD (Fig. 2b, lanes 5 to 8) that was not related to the PAH enzyme, since no corresponding material was present in either rat liver or human liver which contained the native enzyme.

Phenylalanine hydroxylase enzymatic assays (*14*) were performed on extracts from cell lines transformed with MPAH(+) and SV2NEO, control cell lines transformed with SV2NEO alone, and extracts of the human hepatoma cell line hepG-2 (Fig. 3a). No PAH activity was present in NIH3T3 cells or in cells transformed with pSV2NEO alone (Fig. 3a, panel E). Pterin-dependent PAH activity was present in extracts of hepG-2 (Fig. 3a, panel F) and in each of the cell lines stably transformed with MPAH(+) and SV2NEO (Fig. 3a, panels A to D). PAH activity in transformed cells was increased two- to six-fold by induction with $CdCl_2$ (Fig. 3a, panels B and D). No stimulation of activity was found when hepG-2 cells were exposed to $CdCl_2$. The level of activity in the cells transformed with MPAH(+) and SV2NEO was similar to that present in hepG-2 cells prepared and assayed under identical conditions, and was approximately 30-fold less than the activity present in rat liver.

In order to compare the biochemical characteristics of the recombinant enzyme with native human PAH, we determined the apparent Michaelis constant (K_m) values for phenylalanine with crude extracts of the cell line MPN10 (transformed with MPAH(+) and SV2NEO) and the hepG-2 cell line (Fig. 3b). The apparent K_m values for phenylalanine in uninduced MPN10, $CdCl_2$-induced MPN10, and hepG-2 cells were essentially the same (0.15 m*M*).

These data demonstrate that the phPAH247 clone, recombined with a eukaryotic promoter and transferred into cells which do not constitutively produce PAH, expresses mRNA, immunoreactive protein, and enzymatic activity characteristic of native human PAH. This indicates that a single genetic locus, encoding one peptide of 51.9 kD, contains all necessary genetic information for PAH enzymatic activity. Control experiments excluded the possibility that the host NIH3T3 cells contribute any constituents to the activity of the enzyme. Our data provide direct evidence that monomeric subunits of PAH are encoded by a single genetic locus. The results are also consistent with the report that polymorphic forms of the rat enzyme are encoded by independently segregating alleles (*5*).

These results support the validity of using restriction fragment length polymorphisms (RFLP's) for prenatal diagnosis of PKU. We have demonstrated previously that RFLP's at the PAH locus segregate with the disease state in PKU. The fact that one gene codes for the normal enzyme, and that this gene is present in only one copy per haploid genome (*15*), provides the molecular basis for the observation that mutations underlying PKU are consistently linked to a single genetic locus (*8*).

Transformed cell lines expressing human PAH after gene transfer represent a new source of enzyme for biochemical characterization. Similarly, transformed cells containing mutant PAH genes, cloned from affected individuals with PKU, can be used to produce the mutant PAH enzyme for analysis. This method will eliminate the frustrating dependence on rare, and often inadequate, biopsy samples for the characterization of mutant enzymes. Finally, by demonstrating the feasibility of synthesizing the functioning PAH enzyme from recombinant clones, this work provides the groundwork for investigating alternative therapies for PKU by enzyme or gene replacement.

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