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Mouse phenylalanine hydroxylase

Homology and divergence from human phenylalanine hydroxylase

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The laboratory mouse represents an important model for the study of phenylalanine metabolism and the pathochemistry of phenylketonuria, yet mouse phenylalanine hydroxylase (PAH) has not been extensively studied. We report the cloning and sequencing of a mouse PAH cDNA, the expression of enzymic activity from the mouse PAH cDNA clone and the identification of mouse PAH and human PAH by two-dimensional PAGE of liver samples. These data confirm the expected homology of mouse PAH and human PAH and suggest differences in the primary sequence and the phosphorylation state of the two enzymes.

INTRODUCTION

Phenylalanine hydroxylase (PAH, EC 1.14.16.1) has been the object of intensive biochemical investigation ever since Jervis [1] demonstrated that phenylalanine hydroxylation is an essential step in phenylalanine degradation and that this pathway is deficient in phenylketonuria. Rat PAH has been purified by several laboratories and characterized in detail [2–5]. Human PAH has been subject to less intensive study, but, nevertheless, it has also been purified and its essential biochemical properties have been described [6–11]. Many inferences about normal and pathological phenylalanine metabolism in humans arise from studies in rodents [12–14]. The cloning and sequencing of rat PAH [5,15,16] and human PAH [17,18] has demonstrated that these enzymes are highly homologous [18,19].

The laboratory mouse is an increasingly important medium for the study of PAH. Mice treated with pharmacological inhibitors of PAH are used to study the pathophysiology of hyperphenylalaninaemia [20,21], and mutagenesis of mice with ethylnitrosourea has been used to generate an animal model of hyperphenylalaninaemia [22,23]. Nevertheless, mouse PAH has never been purified, and there are few published data describing its properties.

In the present paper we describe cloning and sequencing of a full-length mouse PAH cDNA, comparison of the primary structure of mouse, human, and rat PAHs and constitution of mouse PAH activity in cultured cells. These experiments provide a structural and methodological foundation for characterization of mouse PAH and consideration of the laboratory mouse as a model for human hyperphenylalaninaemia.

MATERIALS AND METHODS

Cloning and sequencing of mouse PAH cDNA

Polyadenylated RNA was prepared from C57B/6J-mouse livers by using the guanidinium thiocyanate method [24] and affinity purification on oligo(dT)–cellulose. cDNAs were size-selected over ā sucrose gradient after first-strand synthesis for cDNA > 1 kb, and a library of 600000 independent recombinants was constructed in bacteriophage λgt11 by using modifications of the method of Young & Davis [25]. The library was probed with the human PAH cDNA (phPAH247) [18] nick-translated to a specific radioactivity of 3×10^8 c.p.m./μg with [^{32}P]dCTP (Amersham), and positive bacteriophages were plaque-purified by secondary and tertiary screening.

The *Eco*RI cDNA insert from the longest clone (moPAH8) was subcloned into M13mp18. Terminal deletions were introduced by *Exo*III digestion [26], and the sequence of overlapping terminally deleted subclones in both orientations was determined by the dideoxy chain-termination method of Sanger *et al.* [27]. This analysis made use of the Protein Identification Resource of the National Biomedical Research Foundation including the programs COMPARE, PRPLOT and CHOFAS.

DNA-mediated gene transfer

The mouse PAH cDNA was cloned into the vector pCEXV [28], which contains the simian virus 40 origin of replication and early-region promoter. A construction containing human PAH cDNA in the vector 91023B [29] has been described previously [30]. These expression constructs were introduced into Cos cells [31] by calcium phosphate co-precipitation [32]. Cells were grown in Dulbecco's modified Eagle's medium (Hazelton) containing 10% (v/v) fetal-calf serum and harvested 48–72 h after transfection.

The PAH assay measures the conversion of [^{14}C]phenylalanine into [^{14}C]tyrosine in the presence of 0.3 mM-phenylalanine, 4×10^5 c.p.m. of [U-^{14}C]phenylalanine (500 mCi/mmol) (Amersham), 0.2 M-potassium phosphate buffer, pH 6.8, 0.15 M-KCl, 6 units of bovine liver catalase (Sigma Chemical Co.), the enzyme sample (maximum protein 500 μg), 2 mM-dithiothreitol and the cofactor 90 μM-6-methyltetrahydropterin (Calbiochem) added sequentially in 100 μl volume and incubated with shaking for 1 h at room temperature [3,11]. The reaction is stopped by incubation in boiling water for 5 min, then in ice for 5 min, and

Abbreviation used: PAH, phenylalanine hydroxylase.

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The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X51942.

centrifugation in an Eppendorf Microfuge for 10 min. A 5 μl portion of each sample was spotted on silica gel 60 (EM) t.l.c. layer chromatography plates and developed by ascending chromatography in chloroform/methanol/conc. NH_3 (11:7:2, by vol.). Phenylanine (R_F 0.75) and tyrosine (R_F 0.40) were located by autoradiography on Kodak XAR film. Activity was quantified by staining with ninhydrin and counting the radioactivity of the tyrosine spot in Aquasol (New England Nuclear).

Immunoreactive PAH was identified by Western blotting with affinity-purified goat anti-(rat PAH) antibody [5] and ^{125}I-labelled pig anti-(goat IgG) antibody after SDS/PAGE and electrophoretic transfer to nitrocellulose paper as described by Johnson *et al.* [33].

Two-dimensional PAGE

The methods used for electrophoresis were those described by O'Farrell [34] as modified by Anderson & Anderson [35,36]. The exact conditions used were those outlined by Dunbar [37]. Liver fragments were homogenized in a solubilization buffer containing 9 M-urea (Bio-Rad Laboratories), 4% (w/v) Nonidet P-40 (Accurate Chemical Co.), 2% (v/v) Ampholines (LKB; 2:1 ratio of pH 3.5–10 and pH 4–6) and 2% (v/v) 2-mercaptoethanol (Bio-Rad Laboratories) for 2 h at 25 °C. Samples were centrifuged at 200000 *g* for 2 h in a Beckman ultracentrifuge (Ti-60 rotor), and 20 μl samples were focused with the use of a 3:1 ratio of pH 3.5–10 and pH 4–6 Ampholines for 12000 V·h in the ISO-apparatus (Health Products). Second-dimension electrophoresis was carried out in 10–20% polyacrylamide slab gels cast by using the DALT casing apparatus (Health Products) as described by Dunbar [37]. Duplicate gels were stained or electrophoretically transferred to nitrocellulose paper. The silver-based colour stain was used to detect proteins [37]. PAH was identified by probing transfer with goat anti-(rat PAH) antibody and ^{125}I-labelled pig anti-(goat IgG) antibody as described above.

RESULTS

Cloning and sequencing of mouse PAH

Ten PAH clones were identified in the mouse liver library. The longest cDNA insert (moPAH8) was subcloned and sequenced in its entirety. A simplified restriction map relative to that of the human PAH cDNA is shown in Fig. 1. This clone comprises 1978 nucleotide residues including a 17-residue poly(A) tail. The probable polyadenylation signal ATAAA starts at position 1965. The first AUG triplet starts at position 58 and is followed by an open reading frame of 1359 nucleotide residues coding for a protein of 453 amino acid residues and a molecular mass of 51786 Da (Fig. 2).

Sequence homology among mouse, human and rat PAHs

The nucleic acid sequences of mouse PAH and human PAH cDNAs are similar within the open reading frame but show no similarity in the 5′ and 3′ untranslated sequences (results not shown). The open reading frame of mouse PAH is confirmed by identity between the predicted amino acid sequences and two peptide fragments of mouse PAH sequenced previously [38,39] (underlined residues in Fig. 2).

The nucleic acid sequences in the open reading frames of mouse PAH and human PAH differ at 179 of 1359 nucleotide residues. 130/179 changes occur in the third codon position, which is consistent with relaxed selection against mutations in this 'wobble' position. 30/179 changes occur in the first codon position and 19/179 changes in the second. C/T or G/A transitions account for 116/179 differences, C/G or G/C (15/179), A/T or T/A (9/179) and G/T or C/A (39/179) transitions being much less common. The nucleic acid sequences of mouse PAH and rat PAH cDNAs [16] show substantial similarity both within the open reading frame and untranslated regions (results not shown).

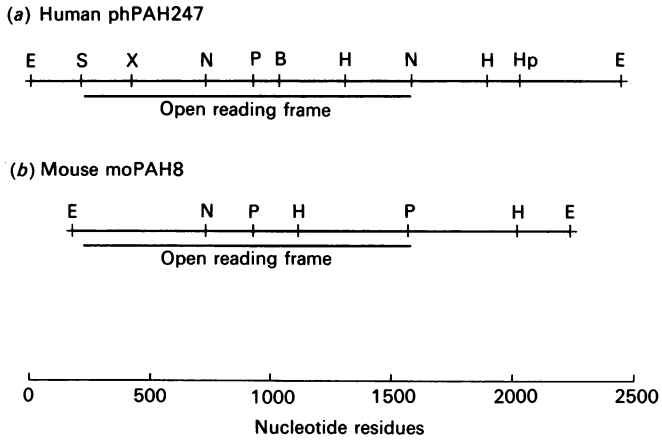

Fig. 1. Schematic structures of human PAH and mouse PAH cDNA clones

(*a*) Restriction map of human PAH (hPAH247) [17]; (*b*) restriction map of mouse PAH (moPAH8). Key: S, *Sma*I; X, *Xba*I; N, *Nco*I; P, *Pst*I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa*I.

The sequence (Fig. 2) of mouse PAH is similar to those of human PAH [28] and rat PAH [16] (Fig. 3). Mouse PAH is the same length as rat PAH, which is one amino acid residue longer than the human protein. There are only ten amino acid differences between rat PAH and mouse PAH, most of which are conservative. There are 36 differences between the human and mouse sequences. Six of the differences occur in the first 12 amino acids, and two-thirds of the differences occur among the first 115 amino acids at the *N*-terminal end. One striking difference between the human and rodent sequences is the reciprocal substitution of serine for cysteine at position 29 and of cysteine for isoleucine at position 373, which preserves the number of cysteine residues (nine).

Expression of recombinant mouse PAH and human PAH in cultured cells

The human PAH cDNA has previously been expressed in Cos cells using the expression vector 91023B [29,30]. The mouse PAH cDNA was subcloned into the eukaryotic expression vector pCEXV and transfected also into Cos cells. At 48–72 h after transfection the cells were harvested, and cytoplasmic extracts were assayed for PAH enzyme activity (Fig. 4*a*) and immunoreactive protein (Fig. 4*c*). PAH enzyme activity was found in extracts of Cos cells transfected with mouse PAH (Fig. 4*a*, lanes 5–8) assayed in the presence of exogenous cofactor (+) but not in extracts assayed in the absence of cofactor (−). The control experiment with the human PAH cDNA is shown (Fig. 4*a*, lanes 1–4). The activity in Cos cells transformed with mouse PAH was higher than in cells transformed with human PAH (Fig. 4*b*). No activity was present in Cos cells transfected with pCEXV as a control (Fig. 4*a*, lanes 9–12). Typical activities in mouse liver and human liver (Fig. 4*a*, lanes 13–16) are also shown.

On Western blotting, a 52 kDa band was observed in human liver (Fig. 4*c*, lane 1), mouse liver (Fig. 4*c*, lane 2), Cos cells transformed with the human PAH (Fig. 4*c*, lane 3) and Cos cells transformed with mouse PAH (Fig. 4*c*, lane 4). No immunoreactive material was present in Cos cells transformed with pCEXV as a control (Fig. 4*c*, lane 5).

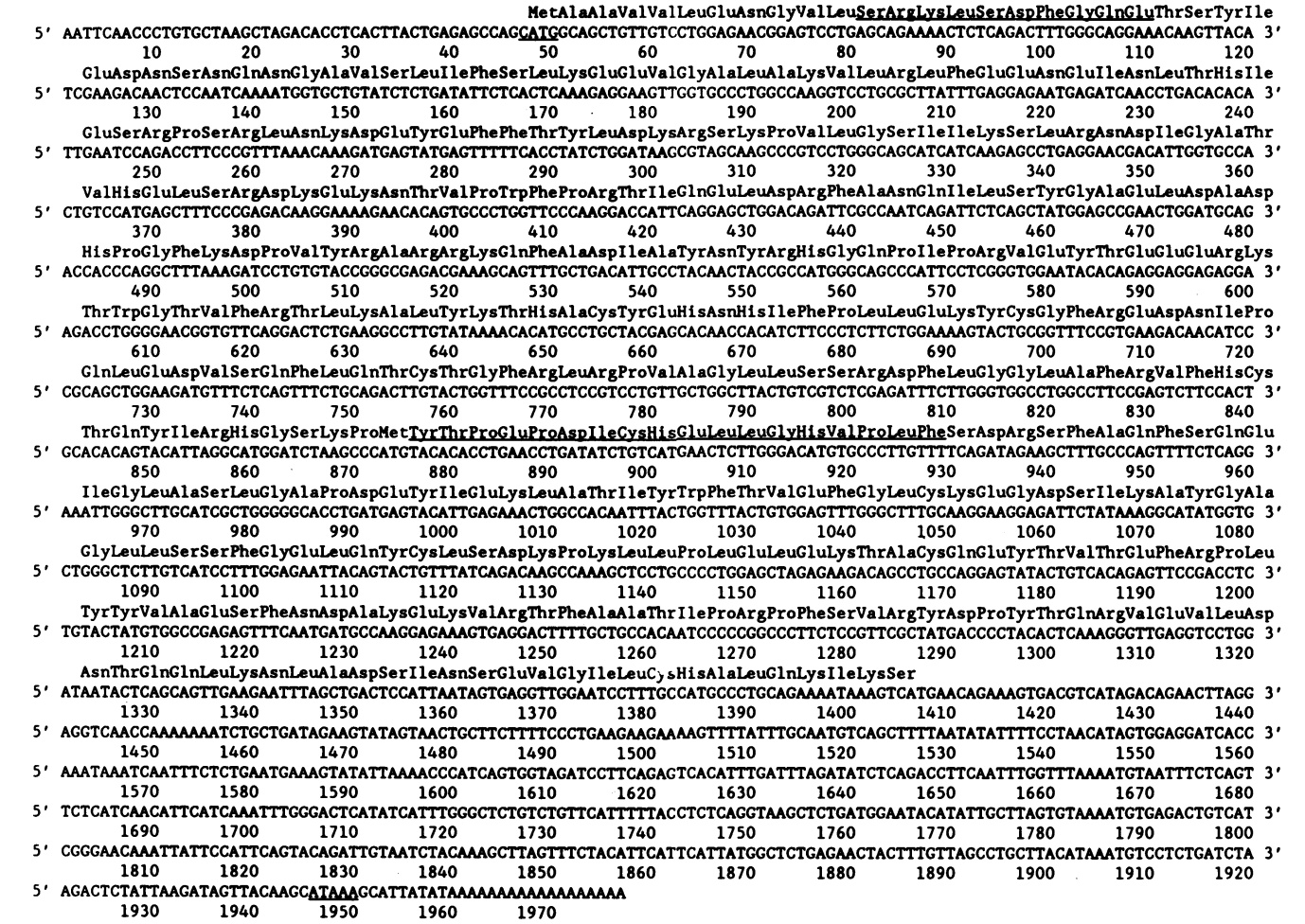

Fig. 2. Nucleic acid sequence of the cDNA (clone moPAH8) and predicted amino acid sequence of mouse PAH

The sequence of moPAH8 cDNA and translation of the long open reading frame are shown. The positions of the polyadenylation signal (ATAAA) and translation initiation codon (ATG) are underlined. Two peptide fragments of rat PAH have been reported that are identical with the underlined residues in the amino acid sequence [38,39].

Identification of PAH by two-dimensional PAGE

Duplicate two-dimensional gels of mouse liver and human liver were prepared for silver staining and Western blotting. A single spot of immunoreactive material was present in human liver, and two adjacent spots were present in mouse liver (results not shown). These spots superimposed with prominent yellow-staining spots indicated by arrows in tri-colour silver stained gels of mouse liver (Fig. 5*a*), human liver (Fig. 5*b*) or a mixture of mouse and human livers (Fig. 5*c*).

DISCUSSION

These experiments describe the cloning and sequencing of a biologically active mouse PAH cDNA. Mouse PAH exhibits little sequence divergence from rat PAH. It also exhibits profound similarity to human PAH, differing at only 36 of 452 amino acid residues. Nevertheless, these studies show several potentially significant differences between mouse PAH and human PAH.

First, most of the differences in the amino acid sequences are clustered within the first *N*-terminal 115 residues. This region of PAH is thought to contain allosteric binding sites for phenylalanine and to constitute an 'inhibitory' domain that regulates the activity of a catalytic domain in the *C*-terminal portion of the molecule [19,40]. Biochemical studies demonstrate that a 35 kDa proteolytic fragment of rat PAH produced by cleaving 11 kDa from the *N*-terminus and 5 kDa from the *C*-terminus retains complete catalytic activity [40]. There are few differences between the mouse PAH and human PAH sequences in this region, and thus it would be predicted that these proteins would have virtually identical catalytic structures. Clusters of amino acid substitutions between mouse (or rat) PAH and human PAH are apparent at the extreme *N*-terminus and in a region adjacent to position 90. Residue 90 is approximately the position where chymotrypic cleavage would remove an *N*-terminal fragment. Thus these sequence changes may occur in a topologically exposed 'hinge' or 'spacer' region between the catalytic and regulatory determinants that is relatively forgiving of evolutionary variation. Nevertheless, since the regulatory function of the *N*-terminal domain is thought to modulate the catalytic domain by physically 'inhibiting' access to catalytic sites, alterations in the structure of this 'hinge' region could significantly alter the natural regulation of catalytic function.

Secondly, there is a reciprocal substitution of cysteine residues between mouse PAH and human PAH at positions 29 and 374. These substitutions preserve the number of cysteine residues but alter their relative positions in the two molecules. These substitutions are significant both because cysteine mutations

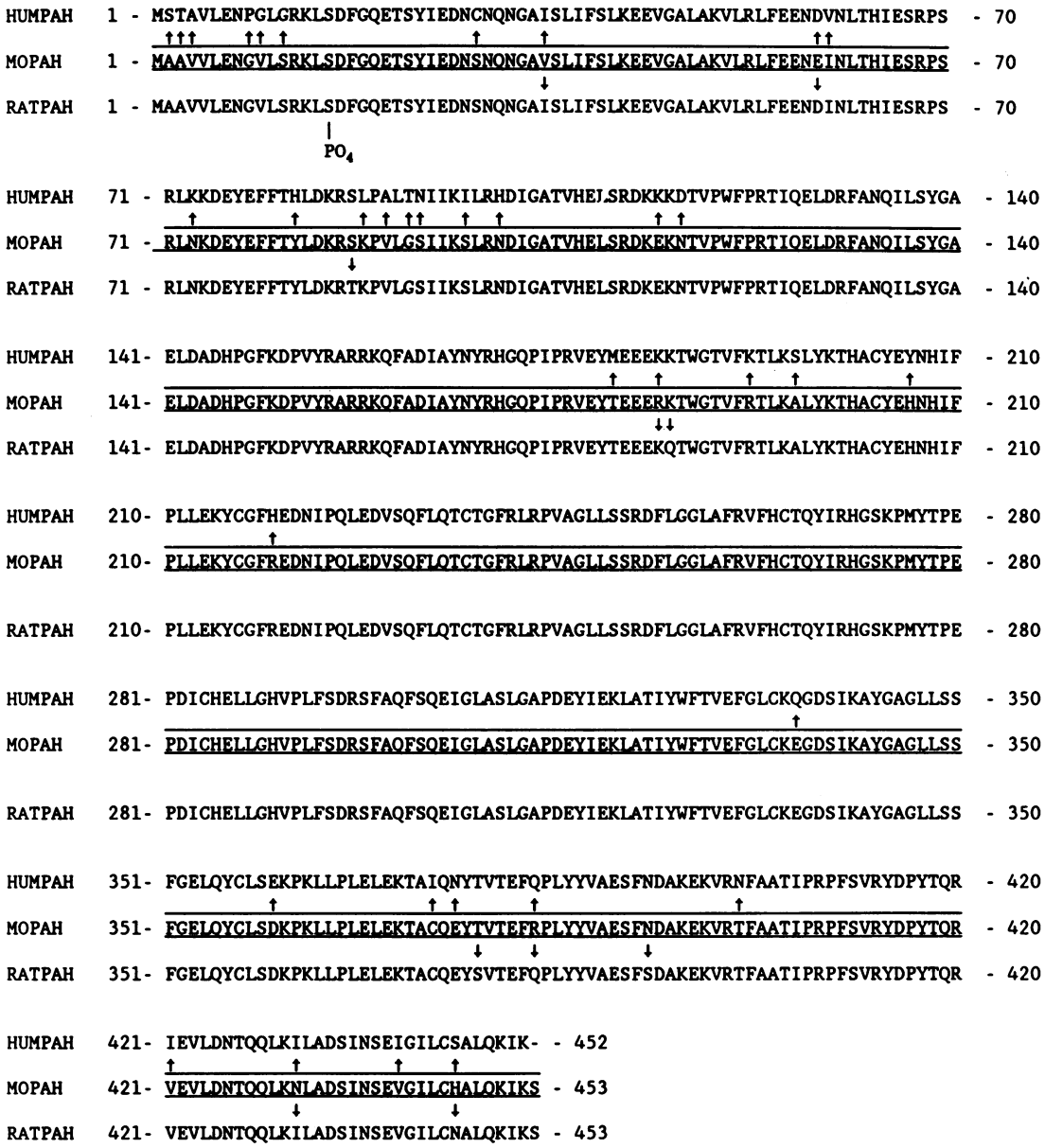

Fig. 3. Alignment of reported PAH sequences from human, mouse and rat

Alignment of mouse PAH (MOPAH), human PAH (HUMPAH) and rat PAH (RATPAH). Residues that are different between mouse PAH and human PAH are indicated by ↑. Residues that are different between mouse PAH and rat PAH are indicated by ↓. The human sequence is from Kwok *et al.* [18]. The rat sequence is from Dahl *et al.* [16]. The position of the phosphorylated serine residue in rat PAH is indicated [38].

are statistically uncommon [41] and because alterations in the topology of the disulphide bonds can potentially alter structure–function determinants. In particular, it has been shown that there are five free thiol groups in rat PAH and that thiol-group modification alters the activation state of the rat enzyme [42]. There are no data indicating which cysteine residues may be involved in this modification.

Thirdly, two-dimensional PAGE analysis reveals two immunoreactive spots of mouse PAH but only one spot of human PAH. The presence of two immunoreactive PAH species noted previously in the rat has been a source of historical controversy. The presence of two bands was initially interpreted as indicative of two physically distinct subunits comprising the PAH multimer. Subsequent studies indicated that the active PAH apoenzyme was constituted from a single gene [43] and that the two bands represented phosphorylated and dephosphorylated forms of PAH protein (reviewed in ref. [6]). Although similar studies have not yet been performed with mouse PAH, it is likely that the two species in mouse liver represent phosphorylated and dephosphorylated forms. Allelic heterogeneity has been observed by PAGE or two-dimensional PAGE in several species [44,45]. The present work was performed in inbred mice in which all loci are presumed to be homozygous; therefore it is unlikely that the two bands observed in the mouse represent allelic heterogeneity.

There remains considerable uncertainty about whether human PAH is a phosphoprotein. Several studies have failed to demonstrate phosphorylation of the human enzyme [10]. Our finding of a single immunoreactive species in human livers by two-dimensional PAGE is consistent with these data. Other studies have suggested that there are multiple species of human PAH on two-dimensional PAGE [46] and that a monoclonal antibody can be raised specifically against the phosphorylated

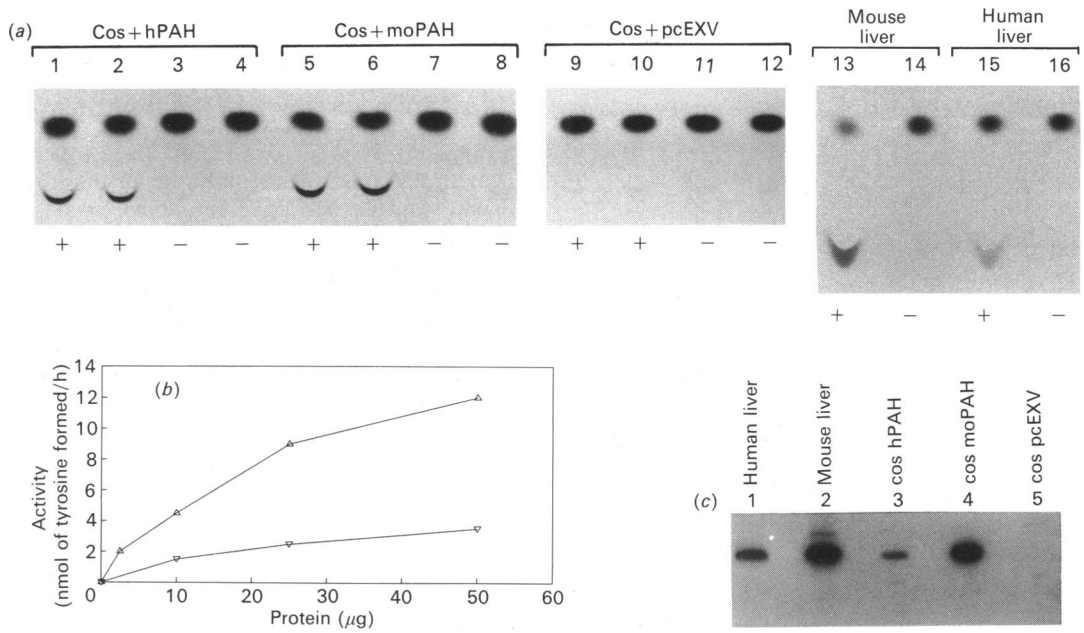

Fig. 4. Expression of PAH in liver and transformed Cos cells

(*a*) PAH enzymic activity in liver, transformed Cos cells and controls. The assay measures the conversion of [^{14}C]phenylalanine into [^{14}C]tyrosine by 250 μg of cellular extracts of 50 μg of human liver in the presence (+) or in the absence (−) of cofactor. Reaction products are separated by t.l.c. and located by autoradiography as shown. (Duplicate samples are shown.) Lanes 1–4, Cos cells transformed with human PAH; lanes 5–8, Cos cells transformed with mouse PAH; lanes 9–12, Cos cells transformed with pCEXV (control vector without PAH insert); lanes 13–16, human liver extract. Panel (*b*) shows that the PAH activity in crude extracts of Cos cells transformed with mouse PAH (△) was higher than the activity in cells transformed with human PAH (▽). (*c*) PAH immunoreactive protein in liver and Cos cells transfected with mouse or human PAH. Portions (100 μg) of liver or cell extracts were separated by SDS/PAGE, electrophoretically transferred to nitrocellulose paper and probed sequentially with affinity-purified goat anti-(rat PAH) antibody and ^{125}I-labelled anti-(goat IgG) antibody. Lane 1, human liver; lane 2, mouse liver; lane 3, Cos cells transformed with mouse PAH expression vector; lane 4, Cos cells transformed with human PAH expression vector; lane 5, Cos cells transformed with pCEXV (control vector without PAH insert).

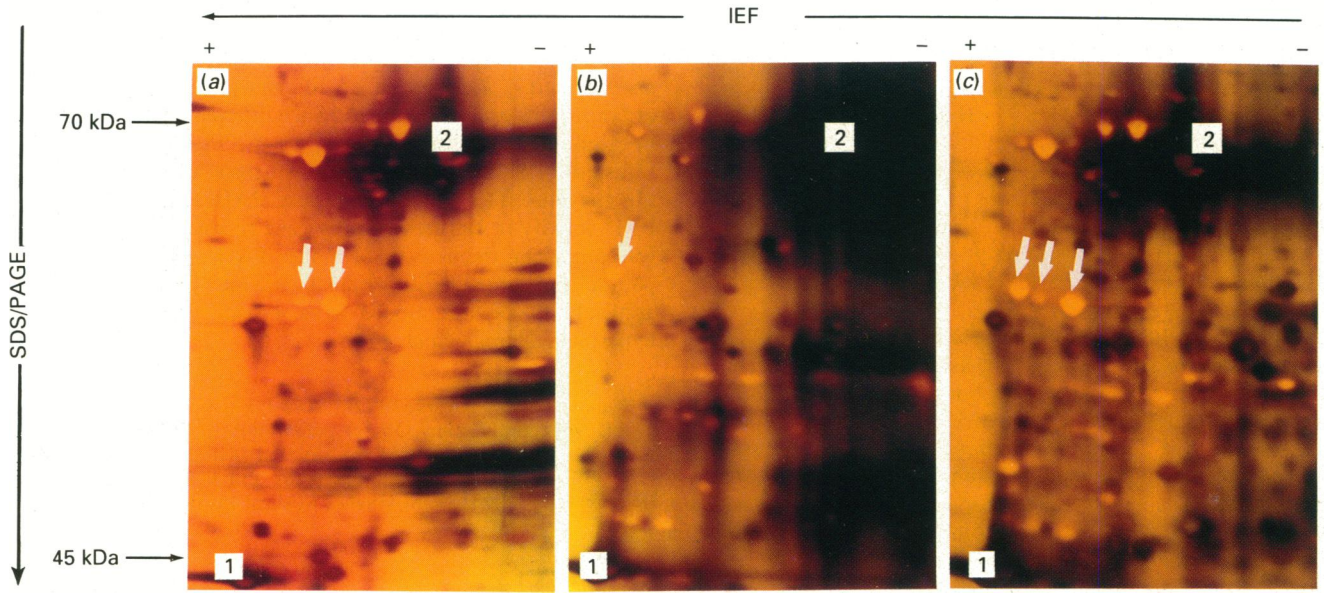

Fig. 5. Identification of PAH on two-dimensional PAGE gels of mouse and human liver

(*a*) Silver-stained two-dimensional gel of mouse liver extract; (*b*) silver-stained two-dimensional gel of human liver extract; (*c*) silver-stained two-dimensional gel of a mixture of mouse and human liver extracts. The identification of PAH spots was made by electrophoretic transfer and immunoblotting of duplicate gels (not shown). Two immunoreactive spots were identified in mouse liver that superimposed with the spots indicated by arrows in panel (*a*). A single immunoreactive spot was identified in human liver that superimposed with the spot indicated by the arrow in panel (*b*). The positions of albumin (1) and β-actin (2) bands are indicated for reference.

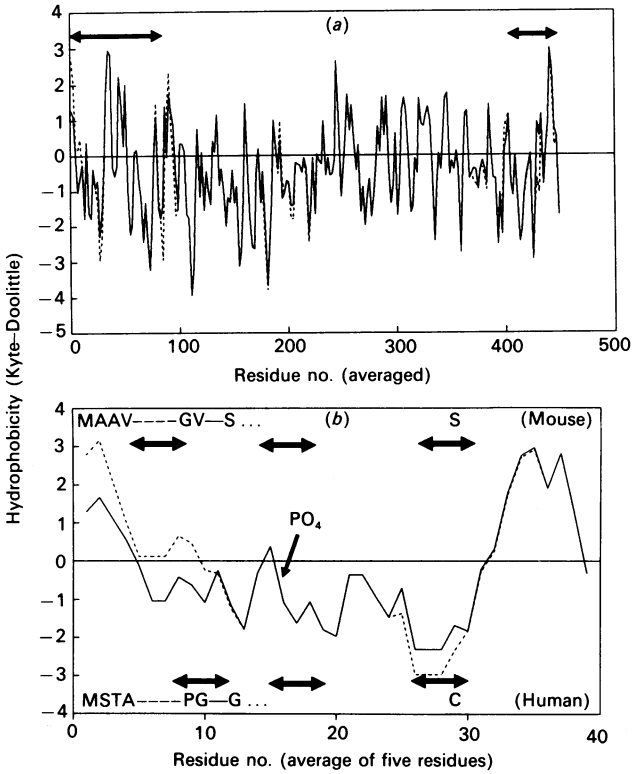

Fig. 6. Predicted hydrophobicity of mouse PAH and human PAH

(*a*) Predicted hydrophobicity of complete human PAH and mouse PAH. Kyte–Doolittle hydrophobicity data [48] were iteratively averaged over five amino acid residues. The approximate positions of the 11 kDa *N*-terminal fragment and 5 kDa *C*-terminal fragment that are removed by proteolysis are indicated by arrows. (*b*) Predicted hydrophobicity of the *N*-termini of mouse PAH and human PAH. Kyte–Doolittle hydrophobicity data are averaged over five residues and graphed as the position of the first residue. The differences in the sequences of mouse PAH and human PAH in this region are noted, as is the position of Ser-16, which is known to be the phosphorylation site in rat PAH. Also indicated (↔) are the positions of potential β-turns predicted by using the Chou–Fasman algorithm. In mouse PAH the β-turn at positions 15–18 has the highest calculated probability, whereas in human PAH the β-turn at positions 8–11 has the highest calculated probability. ——, Human PAH; ------, mouse PAH.

form of human PAH [47]. In the present work we did not observe the multiple forms of human PAH observed by Smith *et al.* [46,47].

The position of the phosphorylated serine residue in rat PAH has been determined to be Ser-16 by isolation and sequencing of a fragment containing the phosphorylated group [38]. The presence of a homologous sequence that maintained the consensus Arg-Lys-Xaa-Ser motif in human PAH suggested that the human enzyme would be similarly phosphorylated [18]. The subsequent sequencing of full-length rat and mouse clones indicates that, although the central motif of the phosphorylation site is preserved, there are seven amino acid differences between the rodent and human sequences in the first 34 amino acids surrounding Ser-16. These include substitutions of Cys-29, Pro-9, Ser-2 and Thr-3 in the human sequence and Ser-29, Gly-9, Ala-2 and Ala-3 in the rodent sequence. The result of these substitutions is to make the *N*-terminal end of human PAH considerably more hydrophilic than those of rodent PAHs (Fig. 6) and to substitute relatively bulky amino acid residues for alamine and glycine. Analysis of potential β-turn sites by using the Chou–Fasman algorithm indicates that, although the sequence in the vicinity of amino acid residues 15–18 encompassing the phosphorylation site would be predicted to be a β-turn in both proteins, the human protein has an even higher potential for β-turn in the vicinity of amino acid residues 8–11 encompassing Pro-9 (Fig. 6*a*). Thus the sequences that could theoretically constitute the substrate for the protein kinase in the human sequence may have an altered topology relative to the rat and mouse sequences and might be less susceptible to phosphorylation.

Previous studies in rats and mice demonstrate that rodents have an order of magnitude greater phenylalanine-hydroxylating activity in their livers than humans. The explanation for this difference remains unclear. In our experiments, more PAH activity was found in mouse liver than in human liver (Fig. 4*a*), lanes 13–26) and more activity was seen in Cos cells transfected with mouse PAH than in cells transfected with human PAH (Fig. 4*b*). There was, however, also correspondingly more immunoreactive protein in the mouse liver and Cos cells transfected with mouse PAH (Fig. 4*c*) and more protein apparent on Coomassie Blue-stained two-dimensional PAGE gels (results not shown), suggesting that the difference in activity might be reflective of the differential steady-state concentrations of the two enzymes in the cells rather than intrinsic differences in apoenzyme structure and function. It is impossible to compare quantitatively the specific activities of the two enzymes from these data, since the anti-PAH antibody raised against rat PAH may not have equal affinity for mouse and human proteins and these enzymes may not have equivalent stability in cellular extracts. This inference, however, is consistent with previous reports, particularly those by Gillam *et al.* [4] and Woo *et al.* [8] indicating that purified rat PAH and human PAH have similar specific activities ($V_{max.}$). Quantitative determination of the relative specific activities of mouse PAH and human PAH will need additional biochemical analysis and purification of these enzymes. This process should be greatly expedited by the ability to express large amounts of active PAH in prokaryotes [11].

Differences in the activity and structure of human and rodent PAHs have important implications for the use of rodents as models for hyperphenylalaninaemia. Differences in activity cannot be merely ascribed to an allometric association between organism size or metabolic rate. Such differences must ultimately be accounted for by differences in the rate of transcription, translation, stability, activation or intrinsic catalytic activity of the enzymes. The cloning of mouse PAH and constitution of PAH activity in cultured cells by gene transfer provide a means for comparing the activity of mouse PAH and human PAH in parallel and controlled experiments and may begin to address the applicability of the mouse as a model system for human hyperphenylalaninaemia.

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