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Genetics

Polysome immunoprecipitation of phenylalanine hydroxylase mRNA from rat liver and cloning of its cDNA

(differential hybridization/hybrid-selected translation/inborn error in metabolism/phenylketonuria/phenylalanine 4-monooxygenase)

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ABSTRACT **The mRNA for phenylalanine hydroxylase (phenylalanine 4-monooxygenase, EC 1.14.16.1) has been purified from total rat liver mRNAs, of which it constitutes less than 0.25%, to greater than 10% purity in a single step by specific polysome immunoprecipitation. The purified mRNA was used for synthesis and cloning of its cDNA. Recombinant colonies containing phenylalanine hydroxylase DNA sequences were identified by differential hybridization, hybrid-selected translation, and blot hybridization analysis. The rat cDNA clone was capable of hybridizing with human phenylalanine hydroxylase mRNA, which will permit the isolation of the corresponding human gene for analysis of phenylketonuria, a hereditary disorder in phenylalanine metabolism that causes permanent mental retardation in humans.**

Phenylketonuria (PKU) is a human disorder caused by an inborn error in aromatic amino acid metabolism. Classical phenylketonuria is characterized by a complete lack of phenylalanine hydroxylase activity [phenylalanine 4-monooxygenase; L-phenylalanine,tetrahydropteridine:oxygen oxidoreductase (4-hydroxylating), EC 1.14.16.1] in the liver (1). The enzyme normally catalyzes the oxidation of phenylalanine to tyrosine, the major metabolic pathway of phenylalanine (2). The lack of this enzymatic activity causes persistent hyperphenylalaninemia and minor metabolic pathways for phenylalanine become overutilized (3, 4). High levels of phenylalanine and its derivatives are toxic and cause disturbances in tyrosine and tryptophan metabolism (5). Diminished formation of catecholamines, melanin, and serotonin is typical in untreated phenylketonuric patients, and the clinical symptom is severe and permanent mental retardation (5). The condition is autosomal recessive and has a prevalence of about 1 in 10,000 (for review, see ref. 6).

Phenylalanine hydroxylase has been purified from livers of rat, monkey, and human (7–9). It is a multimeric enzyme with a subunit molecular weight of about 50,000. Recent evidence has suggested that either the subunits are identical or one is a proteolytic product of the other (10, 11). In order to gain a better understanding of the molecular basis for phenylketonuria at the gene level, we have enriched phenylalanine hydroxylase mRNA from total rat liver polysomes by specific immunoprecipitation and then used the mRNA for the synthesis and cloning of its complementary DNA.

METHODS

Purification of Phenylalanine Hydroxylase and Its Antibody. Rat liver phenylalanine hydroxylase was purified by a modification of the procedure described by Shiman *et al.* (12). Enzyme activity was measured by using the fluorimetric assay for tyrosine (13), and the purity of the enzyme preparation was analyzed by $NaDodSO_4$/polyacrylamide slab gel electrophoresis (14). Purified rat liver phenylalanine hydroxylase was used to immunize a goat, and immunospecificity of the antiserum was analyzed by immunodiffusion (15). Specific immunoglobulin molecules against phenylalanine hydroxylase were purified from the crude antiserum by affinity chromatography using phenylalanine hydroxylase coupled to cyanogen bromide-activated Sepharose (16). The procedure also rendered the purified immunoglobulin preparation free of contamination by ribonucleases.

Purification of Phenylalanine Hydroxylase mRNA by Polysome Immunoprecipitation. Rat liver polysomes were prepared as described (17). Polysomes with nascent chains of phenylalanine hydroxylase were enriched by specific immunoprecipitation using purified anti-phenylalanine hydroxylase immunoglobulin and *Staphylococcus aureus* cells (16). RNA released from the cell pellet by $NaDodSO_4$/EDTA was extracted with phenol/chloroform, and poly(A)-containing RNA was isolated by oligo(dT)-cellulose column chromatography (18). The extent of enrichment for phenylalanine hydroxylase mRNA was estimated by translation of the RNA preparations in an mRNA-dependent cell-free protein-synthesizing system derived from rabbit reticulocytes (19) and immunoprecipitation of radioactive phenylalanine hydroxylase (20). The [^{35}S]methionine and [^{3}H]leucine-labeled protein products were analyzed on $NaDodSO_4$/polyacrylamide gel electrophoresis followed by fluorography (21).

Synthesis and Cloning of Phenylalanine Hydroxylase cDNA. Synthesis of cDNA from purified phenylalanine hydroxylase mRNA and its insertion into the *Pst* I site of pBR322 were essentially as described (22). Cloning was performed according to the guidelines for recombinant DNA research from the National Institutes of Health. Transformants were selected for tetracycline resistance. Recombinants containing rat DNA inserts were identified by using [^{32}P]cDNA synthesized from the enriched phenylalanine hydroxylase mRNA preparation according to the *in situ* hybridization procedure of Grunstein and Hogness (23).

Identification of Phenylalanine Hydroxylase Clones by Differential Hybridization and Hybrid-Selected Translation. Plasmid DNA isolated from recombinants by the mini-lysate procedure (24) was linearized with *Bam*HI and subjected to agarose gel electrophoresis. The DNA was transferred bidirectionally to sheets of nitrocellulose filters (25). Duplicate filters obtained in this manner were allowed to hybridize separately with [^{32}P]cDNAs synthesized from polysomal RNAs enriched for and depleted of phenylalanine hydroxylase mRNA, the latter being poly(A)-containing RNA extracted from polysomes not

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precipitated by the *S. aureus* cells. Plasmid DNAs were prepared from recombinants that were positive in the differential hybridization test and were digested with *Hha* I. The digested DNA was coupled to aminobenzyloxymethylcellulose (26) and allowed to hybridize with total rat liver poly(A)-containing RNA. RNAs eluted from the DNA-cellulose were subsequently analyzed by cell-free translation and immunoprecipitation.

Blot Hybridization Analysis of RNA from Different Tissues. Poly(A)-containing RNAs isolated from various rat and primate tissues were subjected to agarose gel electrophoresis in the presence of formaldehyde (27). The RNA was transferred to nitrocellulose filters (28) and hybridized to a nick-translated [^{32}P]DNA probe (29) in the presence of 10% dextran sulfate. The filter was washed extensively before autoradiography.

RESULTS

Purification of Rat Liver Phenylalanine Hydroxylase and Characterization of Goat Antiserum to Rat Liver Phenylalanine Hydroxylase. The purification of phenylalanine hydroxylase from rat liver was monitored by Coomassie blue staining of 10% polyacrylamide slab gels (Fig. 1). The resulting enzyme preparation contained two bands at 50,000 daltons as reported (7, 10) and appeared to be greater than 95% pure, with an overall purification of about 400-fold. Antibody against rat liver phenylalanine hydroxylase, raised in a goat, was analyzed by double immunodiffusion on an Ouchterlony plate (Fig. 2). The center well contained 25 μl of crude antiserum with increasing amounts of crude rat liver homogenate in wells 1, 3, and 5 and purified phenylalanine hydroxylase in wells 2, 4, and 6. One continuous precipitin band is apparent, indicating that the goat antibody to rat liver phenylalanine hydroxylase is monospecific.

Enrichment of Phenylalanine Hydroxylase mRNA by Polysome Immunoprecipitation. Phenylalanine hydroxylase constitutes about 0.25% of total liver proteins and its mRNA is expected to be present at correspondingly low levels. Considering the fact that the liver synthesizes a whole array of secretory proteins such as serum albumin, which alone constitutes

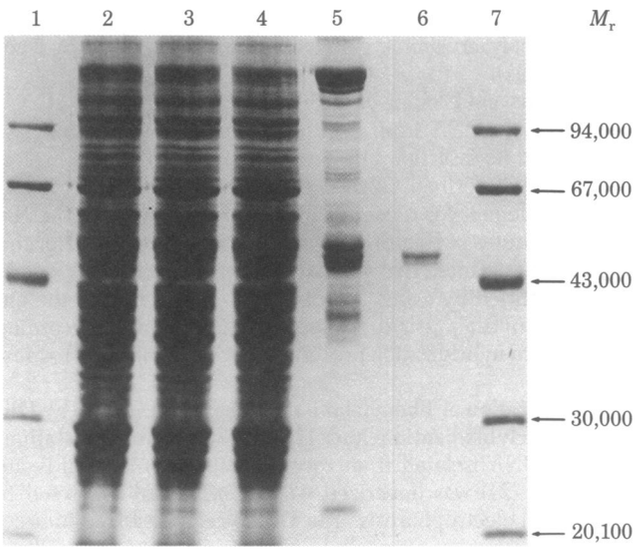

FIG. 1. Purification of rat liver phenylalanine hydroxylase. Various enzyme fractions were analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis (14) followed by Coomassie blue staining. Lanes 1 and 7 contained protein molecular weight standards; lane 2, 250 μg of liver extract; lane 3, 250 μg of liver extract after high-speed centrifugation; lane 4, 250 μg of liver extract after heat activation; lane 5, 80 μg of eluate from the phenyl-Sepharose column; and lane 6, 8 μg of the enzyme after DEAE-cellulose column chromatography (9).

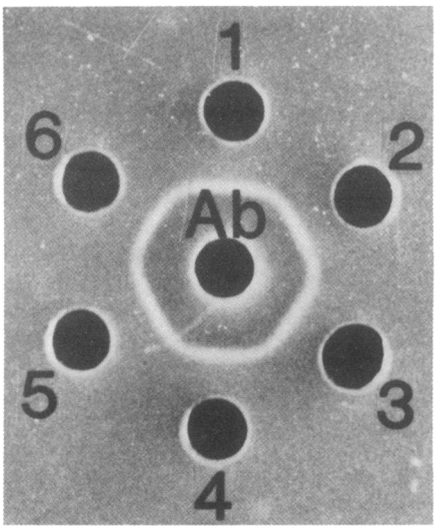

FIG. 2. Double immunodiffusion of goat antiserum to rat liver phenylalanine hydroxylase. The center well contained 25 μl of crude antiserum and peripheral wells contained increasing amounts of crude and purified antigen. Wells 1, 3, and 5 contained 1, 2, and 4 mg of rat liver homogenate, and wells 2, 4, and 6 contained 10, 20, and 40 μg of purified phenylalanine hydroxylase, respectively. The immunodiffusion was allowed to proceed for 20 hr at 25°C.

10–12% of total liver mRNAs, the concentration of phenylalanine hydroxylase mRNA in the liver is likely to be considerably less than its protein concentration. Cell-free translation products directed by total rat liver polysomal RNA were analyzed by NaDodSO$_4$ gel electrophoresis and fluorography; multiple protein bands were observed, with the most prominent one being the 69,000-dalton albumin (Fig. 3*A*, lane 1). Only a minor portion of the protein products was immunoprecipitated with the antibody against phenylalanine hydroxylase, as expected (Fig. 3*B*, lane 1). Polysomes engaged in the synthesis of phenylalanine hydroxylase were enriched by specific polysome immunoprecipitation and poly(A)-containing RNA was isolated from the enriched polysomes. Various RNA fractions eluted from an oligo(dT)-cellulose column were analyzed by *in vitro* translation. It is apparent that a major protein product synthesized in response to the polysome-enriched RNA preparation has a molecular weight of about 50,000 (Fig. 3*A*, lanes 3–6). This protein product was also precipitable with the specific antibody to phenylalanine hydroxylase (Fig. 3*B*, lanes 3–6). The polysome-enriched phenylalanine hydroxylase mRNA preparation appears to be 20% pure, and a greater than 80-fold enrichment from total rat liver mRNA was achieved in a single step.

Construction of the Recombinant Plasmids and Identification of Phenylalanine Hydroxylase Clones by Differential Hybridization. Five micrograms of the polysome-enriched phenylalanine hydroxylase mRNA was employed for cDNA synthesis using reverse transcriptase. Products greater than 1,300 nucleotides selected by alkaline sucrose gradient centrifugation were used to direct the synthesis of the second DNA strand. The enzymatically synthesized DNA was inserted into the *Pst* I site of pBR322, and bacterial transformants were screened by using a [^{32}P]cDNA probe synthesized from polysome-enriched phenylalanine hydroxylase-enriched mRNA (23). Recombinant plasmid DNAs prepared by the mini-lysate procedure were digested with *Bam*HI and resolved by agarose gel electrophoresis. The DNA was transferred bidirectionally to nitrocellulose filters, which were hybridized separately to two [^{32}P]cDNA probes: Fig. 4 *A* and *B* show the radioautograms of the filters after hybridization with [^{32}P]cDNAs synthesized from poly-

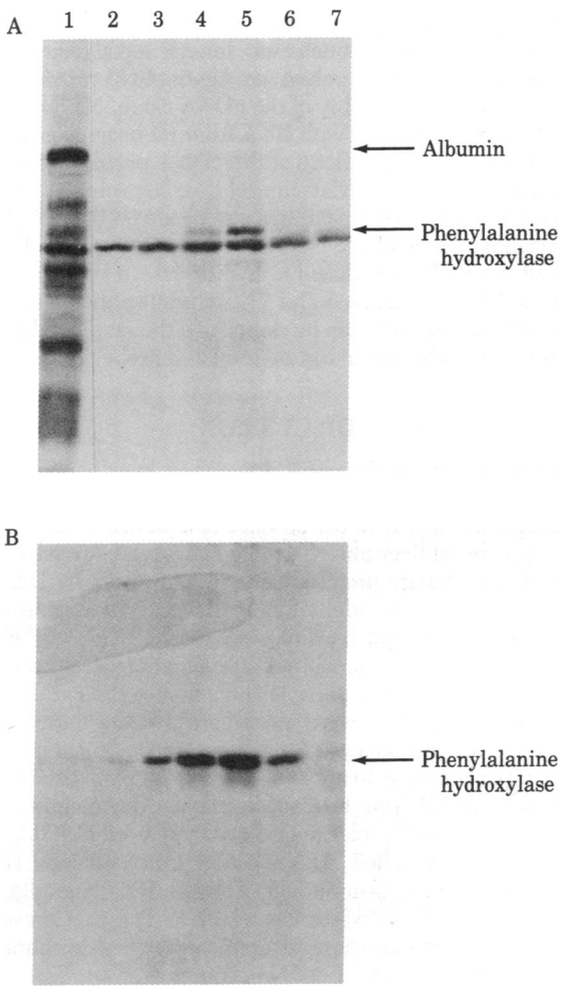

FIG. 3. Enrichment of phenylalanine hydroxylase mRNA by polysome immunoprecipitation as analyzed by cell-free translation followed by $NaDodSO_4$/polyacrylamide gel electrophoresis and fluorography. Total translation products are shown in *A* and products immunoprecipitable with the antibody to phenylalanine hydroxylase are shown in *B*. Lane 1 contained products synthesized in response to total rat liver poly(A)-containing RNA. Lane 2 contained mock-translated products and shows the 50,000-dalton endogenous reticulocyte lysate band. RNAs used for translation in lanes 3–6 were oligo(dT)-cellulose column eluates from the immunoprecipitated polysomes.

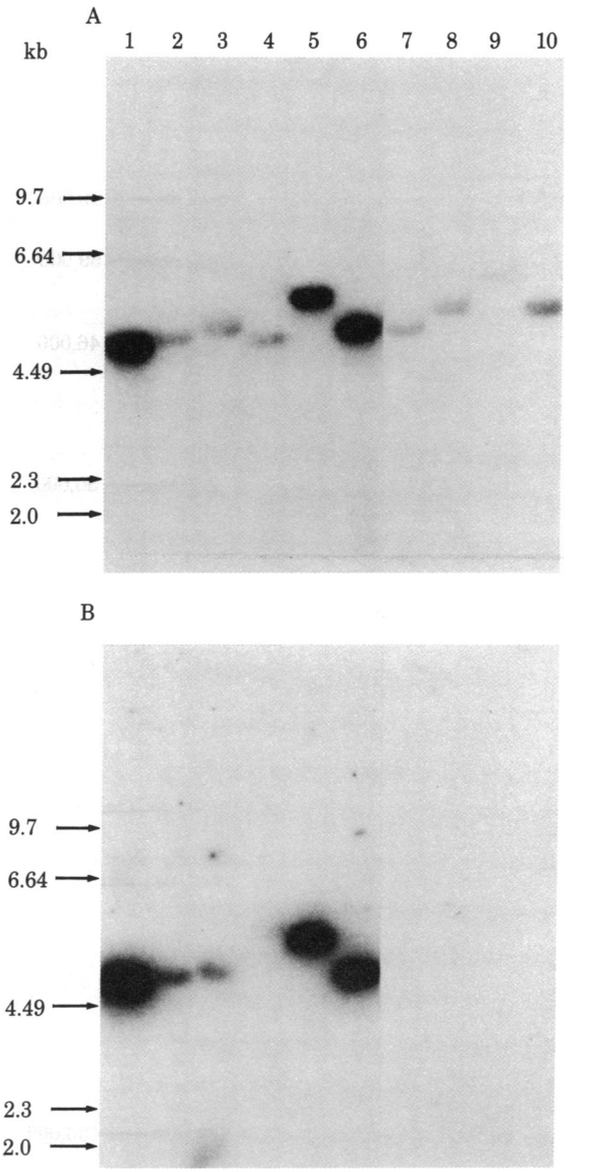

FIG. 4. Identification of candidate phenylalanine hydroxylase cDNA clones by differential Southern hybridization. Recombinant plasmid DNAs prepared from various clones by the mini-lysate procedure (24) were digested with *Bam*HI, resolved by agarose gel electrophoresis, and transferred bidirectionally to nitrocellulose to give duplicate filters. (*A*) Autoradiogram of one filter after hybridization with a [^{32}P]cDNA probe synthesized from polysome-enriched mRNA. (*B*) Autoradiogram of the second filter, which was hybridized with a [^{32}P]cDNA probe synthesized from mRNA depleted of phenylalanine hydroxylase mRNA. kb, Kilobases.

some-enriched mRNA and depleted mRNA preparations, respectively. Differential hybridization signals were observed with several clones (lanes 4, 7, 8, and 10), and they represent candidates to contain phenylalanine hydroxylase DNA sequences. The number of colonies positive in this differential hybridization test represents about 20% of the total number of recombinants. This number correlates well with the estimated purity of phenylalanine hydroxylase mRNA used for cloning.

Confirmation of Phenylalanine Hydroxylase Clones by Hybrid-Selected Translation. Candidate recombinant clones were further analyzed by hybrid-selected translation. Plasmid DNAs isolated from these colonies were denatured and coupled to aminobenzyloxymethylcellulose. The immobilized DNA was allowed to hybridize with total rat liver poly(A)-containing RNA. RNAs eluted from the columns were translated *in vitro* and total protein products were analyzed by electrophoresis on $NaDodSO_4$/polyacrylamide gels followed by fluorography. In this experiment, the 50,000-dalton band endogenous to the reticulocyte lysate system was selectively removed by boiling the gel in 10% trichloroacetic acid (M. R. Waterman, personal communication). Products synthesized in response to mRNA selected by the cloning vector pBR322 DNA as the negative control are shown in Fig. 5*A*, lane 1. The profile is similar to that of total rat liver poly(A)-containing RNA and very little, if any, of the product is immunoprecipitable with the specific antibody against phenylalanine hydroxylase (Fig. 5*B*, lane 1). As positive controls for hybrid selection, two independent rat cDNA clones coding for two smaller liver proteins were included in the test, and two intense bands were obtained (Fig. 5*A*, lane 2), with no immunoprecipitable phenylalanine hydroxylase (Fig. 5*B*, lane 2). Protein products synthesized in response to RNA selected by the candidate clones, however, contained a major band at 50,000 daltons, suggesting the presence of phenylalanine hy-

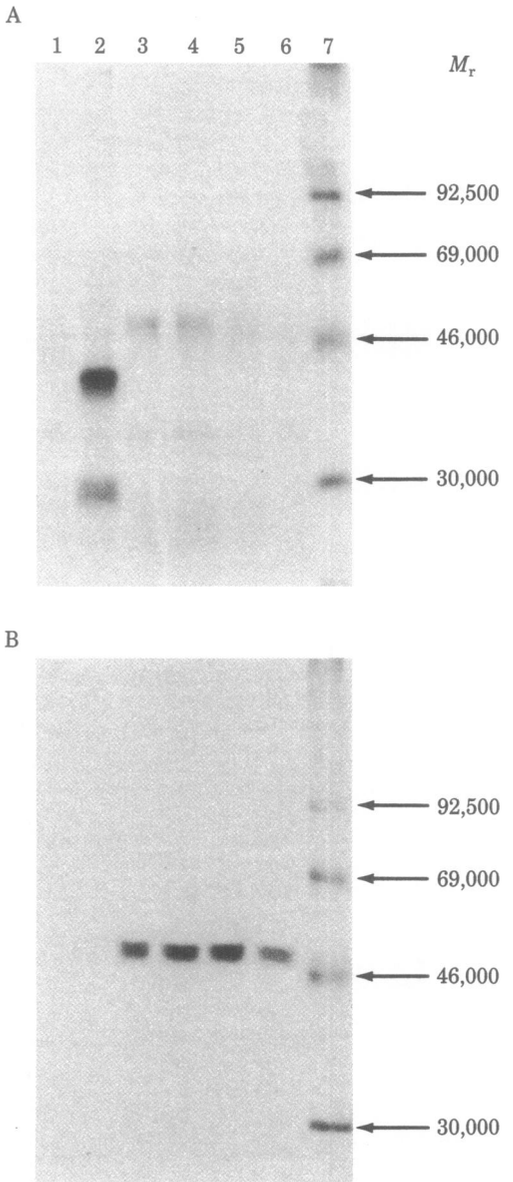

FIG. 5. Identification of phenylalanine hydroxylase cDNA clones by hybrid-selected translation. DNAs from the candidate recombinant clones were covalently coupled to aminobenzyloxymethylcellulose and used to enrich corresponding mRNA species from total rat liver poly(A)-containing RNA by affinity hybridization. Bound RNAs were eluted thermally and analyzed subsequently by cell-free translation. Total translation products are shown in *A* and the products immunoprecipitated with the antibody to phenylalanine hydroxylase are shown in *B*. DNAs used for hybrid-selection were pBR322 in lane 1; two independent rat liver cDNA clones coding for two smaller rat liver proteins in lane 2; prPH98 in lane 3; prPH92 in lane 4; prPH91 in lane 5; and prPH86 in lane 6. Lane 7 in *A* and *B* contained size markers.

droxylase (Fig. 5*A*, lanes 3–6). That these protein bands are precipitable in their entireties with the specific antibody to phenylalanine hydroxylase (Fig. 5*B*, lanes 3–6) indicate that these clones do contain phenylalanine hydroxylase DNA sequences.

RNA Analysis by Blot Hybridization. Total poly(A)-containing RNAs were extracted from various rat and primate tissues and their phenylalanine hydroxylase mRNA contents were analyzed using a rat liver phenylalanine hydroxylase cDNA clone as the probe (Fig. 6). A hybridizing molecule of about 21S was obtained for total rat liver poly(A)-containing RNA (lane 2). The fact that 0.5 μg of polysome-enriched phenylalanine hydroxylase mRNA generated a much more intense signal (lane 1) than did 5 μg of total rat liver poly(A)-containing RNA provided further support for the fidelity of the cDNA clone. No hybridization signal was obtained with RNA from rat heart (lane 3), indicating the tissue specificity of the cDNA probe. Rat kidney is known to contain phenylalanine hydroxylase activity (30), and a corresponding mRNA band was also observed (lane 5). More importantly, the rat cDNA clone (prPH98) was capable of cross-hybridizing with phenylalanine hydroxylase mRNA from baboon and human (lanes 6 and 7), suggesting that the corresponding human genes can be cloned and the rat phenylalanine hydroxylase cDNA clone can be used as a probe.

DISCUSSION

This report outlines the purification of rat liver phenylalanine hydroxylase mRNA by polysome immunoprecipitation and the cloning of its cDNA. In the absence of a published amino acid sequence for rat liver phenylalanine hydroxylase we have been denied the ultimate proof of clone identification by DNA sequence analysis. The fidelity of the cDNA clone was provided by three independent lines of evidence: (*i*) Using [^{32}P]cDNA probes synthesized from mRNA preparations that were enriched and depleted for phenylalanine hydroxylase, differential hybridization signals were generated by the bidirectional Southern blotting analysis. (*ii*) These recombinant plasmids were capable of selectively hybridizing with phenylalanine hydroxylase mRNA from total rat liver poly(A)-containing RNA. (*iii*) RNA analysis by blot hybridization showed that 0.5 μg of the polysome-enriched mRNA preparation contained significantly more phenylalanine hydroxylase mRNA than did 5 μg of total rat liver poly(A)-containing RNA. Together these data strongly suggest that these recombinant clones do contain rat phenylalanine hydroxylase DNA sequences.

When blot hybridization analysis was carried out with mRNA preparations from different tissues, the rat phenylalanine hydroxylase cDNA clone (prPH98) was able to hybridize with RNA from rat liver, rat kidney, rat adrenal, baboon liver, and human liver, but not rat heart, and baboon kidney. Hybridization signals were obtained from the liver samples as expected. Phenylalanine hydroxylase activity has been reported in the kidneys of rats (30) but not primates (31). The detection of phenylalanine hydroxylase mRNA in the rat kidney but not the baboon kidney is thus completely consistent with these observations. Interestingly, a hybridization band of identical molecular weight was also obtained from rat adrenal RNA (Fig. 6, lane 4). Although the adrenal does not contain phenylalanine hydroxylase, it does contain tyrosine hydroxylase (EC 1.14.16.2), which converts L-tyrosine to L-dihydroxyphenylalanine (L-dopa), which is the first step in the biosynthesis of catecholamines (32). The facts that the two enzymes use the same cofactor (tetrahydropteridine) for hydroxylation of their substrates and that antibody to one crossreacts with the other (33) would suggest that the hybridization band in rat adrenal RNA might represent tyrosine hydroxylase mRNA, which can be cloned by using the phenylalanine hydroxylase cDNA clone as a probe.

It has been reported that antibody to rat liver phenylalanine hydroxylase crossreacts with the human enzyme with complete fusion of the immunoprecipitation lines (33), indicating that the human and rat enzymes exhibit a certain degree of structural and sequence homology. In light of the observation that the rat cDNA clone cross-hybridized with human phenylalanine hydroxylase mRNA, it will be possible to clone the human phenylalanine hydroxylase gene by using the rat cDNA clone as a probe. The availability of the human gene for phenylalanine

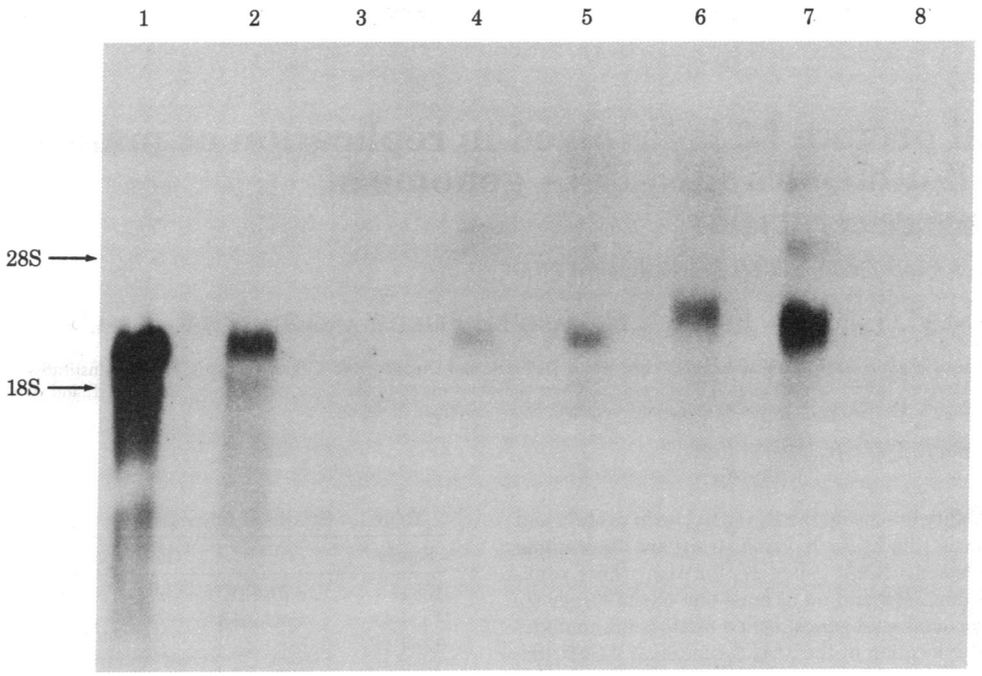

FIG. 6. Blot hybridization of labeled prPH98 DNA with total poly(A)-containing RNA from various tissues. Lane 1 contained 0.5 μg of polysome-enriched phenylalanine hydroxylase mRNA; lane 2 contained 5 μg of rat liver RNA; lane 3, 25 μg of rat heart RNA; lane 4, 25 μg of rat adrenal RNA; lane 5, 5 μg of rat kidney RNA; lane 6, 25 μg of human liver RNA; lane 7, 25 μg of baboon liver RNA; and lane 8, 25 μg of baboon kidney RNA.

hydroxylase will permit the analysis at the molecular level of classical phenylketonuria and various types of hyperphenylalaninemia. Recently the cloned human α- and β-globin genes have been used to develop methods of prenatal diagnosis for hereditary disorders such as the thalassemias and sickle cell anemia (34, 35). Similar studies using the human phenylalanine hydroxylase gene should also permit the development of gene mapping methods for prenatal diagnosis of phenylketonuria.

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