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Genetics

Regional mapping of the phenylalanine hydroxylase gene and the phenylketonuria locus in the human genome

(somatic cell hybrid/*in situ* hybridization/human chromosome 12)

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ABSTRACT **Phenylketonuria (PKU) is an autosomal recessive disorder of amino acid metabolism caused by a deficiency of the hepatic enzyme phenylalanine hydroxylase (PAH; phenylalanine 4-monooxygenase, EC 1.14.16.1). A cDNA clone for human PAH has previously been used to assign the corresponding gene to human chromosome 12. To define the regional map position of the disease locus and the *PAH* gene on human chromosome 12, DNA was isolated from human–hamster somatic cell hybrids with various deletions of human chromosome 12 and was analyzed by Southern blot analysis using the human cDNA *PAH* clone as a hybridization probe. From these results, together with detailed biochemical and cytogenetic characterization of the hybrid cells, the region on chromosome 12 containing the human *PAH* gene has been defined as 12q14.3→qter. The *PAH* map position on chromosome 12 was further localized by *in situ* hybridization of ^{125}I-labeled human *PAH* cDNA to chromosomes prepared from a human lymphoblastoid cell line. Results of these experiments demonstrated that the region on chromosome 12 containing the *PAH* gene and the PKU locus in man is 12q22→12q24.1. These results not only provide a regionalized map position for a major human disease locus but also can serve as a reference point for linkage analysis with other DNA markers on human chromosome 12.**

Phenylalanine hydroxylase (PAH; phenylalanine 4-monooxygenase, EC 1.14.16.1) converts phenylalanine to tyrosine, using tetrahydrobiopterin as a cofactor. The enzyme is synthesized in the liver, and a variety of deficiency syndromes causing various levels of hyperphenylalaninemia have been observed in man. Reduced activity of PAH results in elevated serum levels of phenylalanine and other normally minor metabolites. Severe PAH deficiency, known as classical phenylketonuria or PKU, causes severe mental retardation in untreated patients (1, 2). PKU is transmitted as an autosomal recessive trait, with a prevalence of about 1 in 10,000 among Caucasians. Newborn screening to detect PKU is mandated by law in most western countries, and therapeutic diets that restrict phenylalanine intake can dramatically improve the prognosis of individuals with PKU. Although the diet must be rigidly implemented throughout the first decade of life to be effective, it has set the precedent for contemporary approaches to the clinical management of inborn errors of metabolism (see refs. 3–5 for review). Since the enzyme is expressed in the liver and not in fibroblast cells, conventional methods of prenatal diagnosis of metabolic disorders by assaying specific enzyme functions in cultured amniocytes cannot be applied in PKU. The investigation into the molecular basis of PKU has begun with the cloning of the cDNAs for rat and human PAH, from which the complete primary structure of the human enzyme has been deduced (6–8). The cloned cDNA has been used to identify multiple restriction site polymorphisms at the human *PAH* locus, which can be used to trace the transmission of the mutant alleles in PKU families (8, 9). This procedure has recently been applied to prenatal diagnosis in PKU families at risk by fetal DNA analysis. The diagnoses were confirmed by the observation that the neonates were of the predicted phenotypes (10).

The assignment of the PKU locus in man has previously been attempted by a number of laboratories by analysis of linkage with other known genetic loci. Because the disorder is autosomal recessive and the pedigrees analyzed were generally not very extensive, these studies have led to conflicting and inconclusive results (11–15). We have recently reported the use of a cloned human *PAH* cDNA probe to assign the corresponding gene to human chromosome 12, using human–mouse hybrids in molecular hybridization experiments (16). In this paper, we describe the use of human–hamster cell hybrids containing various human chromosome 12 deletions for regional mapping of the *PAH* gene. The results were confirmed and further refined by *in situ* hybridization using radioactively labeled human PAH cDNA probes to metaphase chromosome preparations of a human lymphoblastoid cell line of normal karyotype.

MATERIALS AND METHODS

Cell Hybrids and Cell Culture. The human–hamster somatic cell hybrids were constructed by different methods. The hybrid E4E (previously designated 12A), which contains the entire genome of the Chinese hamster ovary glycine-requiring cell mutant CHO-K1/glyA and a single human chromosome 12, has been previously described (17, 18). The cell hybrids 37A9 (previously designated A9 and 12A-1) and 45-3C (previously designated MA2 and 12A-2) were derived from hybrid 12A after treatment with 5-bromodeoxyuridine and near visible light or x-rays (18, 19). The hybrids 16-33, 16-16, and 23-47 were isolated after fusion between 5-bromodeoxyuridine- and near visible light-treated normal human fibroblasts and CHO-K1/glyA cells (18). The hybrid 60A2 contains only a small centromeric fragment of human chromosome 12 and has also been described (20). The culture conditions for the hybrids have been previously described (18).

Cytogenetic and Isozyme Analyses. The methods for chromosome banding and isozyme marker assays are the same as already described (17, 18). Confirmation of the human chro-

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Abbreviations: PAH, phenylalanine hydroxylase; PKU, phenylketonuria; kb, kilobase(s).

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mosomes, either intact or partial, in the hybrids was done by using the sequential staining procedure involving both trypsin banding and Giemsa-11 differential staining techniques performed on the same metaphase spread but in sequential steps (21).

Southern Blotting Analysis. Genomic DNAs were extracted from hybrid cells by previously described methods (8). DNA was digested with *Bam*HI (New England Biolabs) according to the supplier's specifications. Cleaved DNA fragments were separated according to size on 0.8% agarose gels by electrophoresis at 50 V for 20 hr. The *Eco*RI insert of phPAH247, containing the full-length cDNA for human PAH (7), was labeled with ^{32}P by nick-translation and used as the hybridization probe. Methods for transfer of DNA to nitrocellulose filter and conditions for hybridization, washing, and autoradiography have been described (8).

***In Situ* Hybridization.** Metaphase chromosome spreads from human lymphoblastoid cells were G-banded and photographed before hybridization.

The probe used was phPAH247, a recombinant plasmid containing a full-length human *PAH* cDNA insert (7), which was labeled by nick-translation with 5-[^{125}I]iododeoxycytidine triphosphate (Amersham) to a specific radioactivity of 3.4×10^8 dpm/μg.

Chromosomes were hybridized at 39°C for 16 hr at a probe concentration of 0.15 μg/ml, after which coverslips were floated off the slides in 2× NaCl/Cit (1× NaCl/Cit = 0.15 M NaCl/0.015 M sodium citrate)/50% (vol/vol) formamide/0.1 M KI at 37°C for 1 hr and then rinsed in NaCl/Cit followed by 0.5× NaCl/Cit, both at 50°C. After a final wash in 0.5× NaCl/Cit overnight at room temperature, the slides were dehydrated through an ethanol series and air dried. The slides were autoradiographed by using Kodak NTB emulsion (7- to 21-day exposures) and stained with 0.075% Wright's stain in 60 mM phosphate buffer at pH 6.8 (22).

RESULTS

Southern Blot Analysis of Hybrid Cell DNA. To perform regional mapping of the *PAH* gene, human–hamster hybrid cell lines containing various parts of human chromosome 12 were used for hybridization studies. Since the hybrids used in this study were derived from hamster cells, many of which contain a complete hamster genome, it is important to establish differential hybridization patterns between the human and hamster *PAH* genes. Genomic DNAs isolated from normal human lymphocytes and CHO-K1 cells were digested by *Bam*HI and analyzed by Southern blot analysis, using a full-length human PAH cDNA clone (7) as the hybridization probe. The human *PAH* gene is a single-copy gene containing multiple introns (A. G. DiLella, personal communication) and there were five hybridizing *Bam*HI fragments, 20.5, 12.0, 9.4, 8.7, and 5.5 kilobases (kb) in length (Fig. 1, lane 1). The human cDNA also hybridized with two fragments, 25 and 8 kb in length, from the hamster *PAH* gene (Fig. 1, lane 2), but the pattern can be easily distinguished from that of the human *PAH* gene. DNAs isolated from human–hamster cell hybrids containing all or various portions of human chromosome 12 were digested with *Bam*HI and analyzed for the presence or absence of the human *PAH* gene. All seven hybrids contained the two fragments corresponding to the hamster *PAH* gene as expected (Fig. 1, lanes 3–9). DNAs isolated from the hybrids E4E, 37A9, and 16-33 displayed the hybridization pattern corresponding to the human *PAH* gene (Fig. 1, lanes 3, 4, and 8), indicating that these three hybrids must contain the region of human chromosome 12 in which the *PAH* gene resides. The other four hybrids, 45-3C, 60A2, 16-16, and 23-47, failed to show hybridization bands corresponding to the human gene (Fig. 1, lanes 5, 6, 7, and 9), indicating that the chromosomal 12 regions deleted in these hybrids should contain the *PAH* gene.

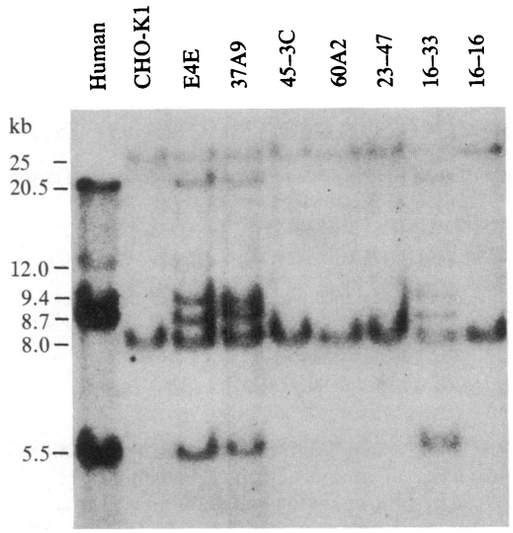

FIG. 1. Southern blot analysis of total *Bam*HI-digested genomic DNA, using labeled human *PAH* cDNA as the hybridization probe. Shown are DNA isolated from normal human lymphocytes in lane 1; Chinese hamster ovary cell line CHO-K1 in lane 2; and the following hybrid cell lines: E4E in lane 3; 37A9 in lane 4; 45-3C in lane 5; 60A2 in lane 6; 23-47 in lane 7; 16-33 in lane 8; and 16-16 in lane 9.

Isozyme Analysis of Hybrid Cells. The genes coding for the enzymes triose phosphate isomerase 1 (*TPI-1*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), lactate dehydrogenase B (*LDHB*), enolase 2 (*ENO2*), serine hydroxymethyltransferase (*SHMT*), and peptidase B (*PEPB*) have all been regionally mapped on chromosome 12 and are useful markers in the analysis of chromosome 12 deletions (19, 23). The results of isozyme analysis for each of the hybrid cell lines used in the hybridization study are given in Table 1. The short arm of chromosome 12 was excluded as the region for the *PAH* gene because hybrids 45-3C and 16-16 contained the short arm markers *TPI-1*, *GAPDH*, and *LDHB* but were negative for the *PAH* gene. The centromeric region and the *SHMT* locus, q12–q14, could also be excluded with hybrids 16-16 and 23-47, both of which were positive for the *ENO2* and the *SHMT* loci, which flank the centromere, but negative for *PAH*. Syntenic analysis of the presence of the *PAH* gene and the presence or absence of each isoenzyme marker revealed concordant segregation with the *PEPB* gene marker, which resides at 12q21 (19). Thus, by isozyme marker analysis of the hybrids, the human *PAH* gene can be mapped to a region of the long arm of chromosome 12 distal to the *SHMT* locus.

Cytogenetic Analysis of Hybrid Cells. Cytogenetic analysis of the hybrids by chromosome banding was next performed on the hybrid cells, and the results illustrating the deleted regions of human chromosome 12 in each hybrid cell line are also shown in Table 1. The hybrid E4E, which contained a complete human chromosome 12 and no other human chromosomal material, was scored positive for the human *PAH* gene. This result confirmed our previous assignment of the human *PAH* gene to chromosome 12 (16). The hybrid cell line 37A9 contained a partially deleted short arm covering the region pter→p1205 and was positive for the *PAH* gene. Cytogenetic analysis of 45-3C showed that it contained the entire short arm plus the proximal portion of the long arm, including the band q12, while that of 60A2 showed the presence of a centromeric region of chromosome 12. Since both hybrids lacked the human *PAH* gene, these regions can be excluded as the location of the *PAH* gene. The critical

Table 1. Regional mapping of the *PAH* gene on human chromosome 12 by molecular hybridization using human–hamster hybrid cells containing partially deleted human chromosome 12

Hybrid cell line	Isozyme markers and positions on chromosome 12							Cytogenetic analysis of chromosome 12
	TPI p13	*GAPDH* p13	*LDHB* p12.1–p12.2	*ENO2* p11–12	*SHMT* q12–q14	*PEPB* q21	*PAH*	
E4E	+	+	+	+	+	+	+	Chromosome 12 intact
37A9	−	−	−	−	+	+	+	pter→p1205 deleted
45–3C	+	+	+	+	−	−	−	q12→qter deleted
60A2	−	−	−	−	+	−	−	Only centromeric fragment present
23–47	−	−	+	+	+	−	−	Not determined
16–33	−	−	−	+	+	+	+	Long arm intact
16–16	+	+	+	+	+	−	−	q14.3→qter deleted

hybrid was 16-16, which was negative for the *PAH* gene and contained a deletion of the distal portion of the long arm. Detailed karyotyping of this hybrid showed that the breakpoint on chromosome 12 resides within the q14 band and occurred at q14.3. The cytogenetic data agree well with the biochemical analysis, and the human *PAH* gene can therefore be mapped to the region q14.3→qter on chromosome 12.

***In Situ* Hybridization.** To further define the chromosomal location of the *PAH* gene, we performed *in situ* hybridization, using chromosome preparations from a karyotypically normal Epstein–Barr virus-transformed human lymphoblastoid cell line. The chromosomes were banded and photographed either prior to hybridization or after autoradiography. The former approach permits unambiguous identification of all chromosomes and precludes bias in the data analysis, since the only criterion applied for selection of metaphase spreads is that they be of good cytologic quality. A representative chromosome spread hybridized with the ^{125}I-labeled probe is shown in Fig. 2. A discrete accumulation of silver grains is detected on both homologs of chromosome 12. The distribution of silver grains observed over 100 previously photographed spreads was plotted on a histogram in which a standardized idiogram of the haploid human genome (24) was divided into units scaled to the average diameter of a silver grain (0.35 μm). In total, 243 grains were associated with chromosomes, of which 90 (37%) were located on chromosome 12 (Fig. 3*A*). An average accumulation of 1 grain per unit chromosome length was observed as background. Analysis of the distribution of silver grains detected on chromosome 12 revealed that 81 (90%) were localized to 12q22→q24.1 (Fig. 3*B*). Statistical evaluation by Poisson distribution of the number of grains per unit chromosome length indicated a highly significant accumulation ($P < 1.3 \times$

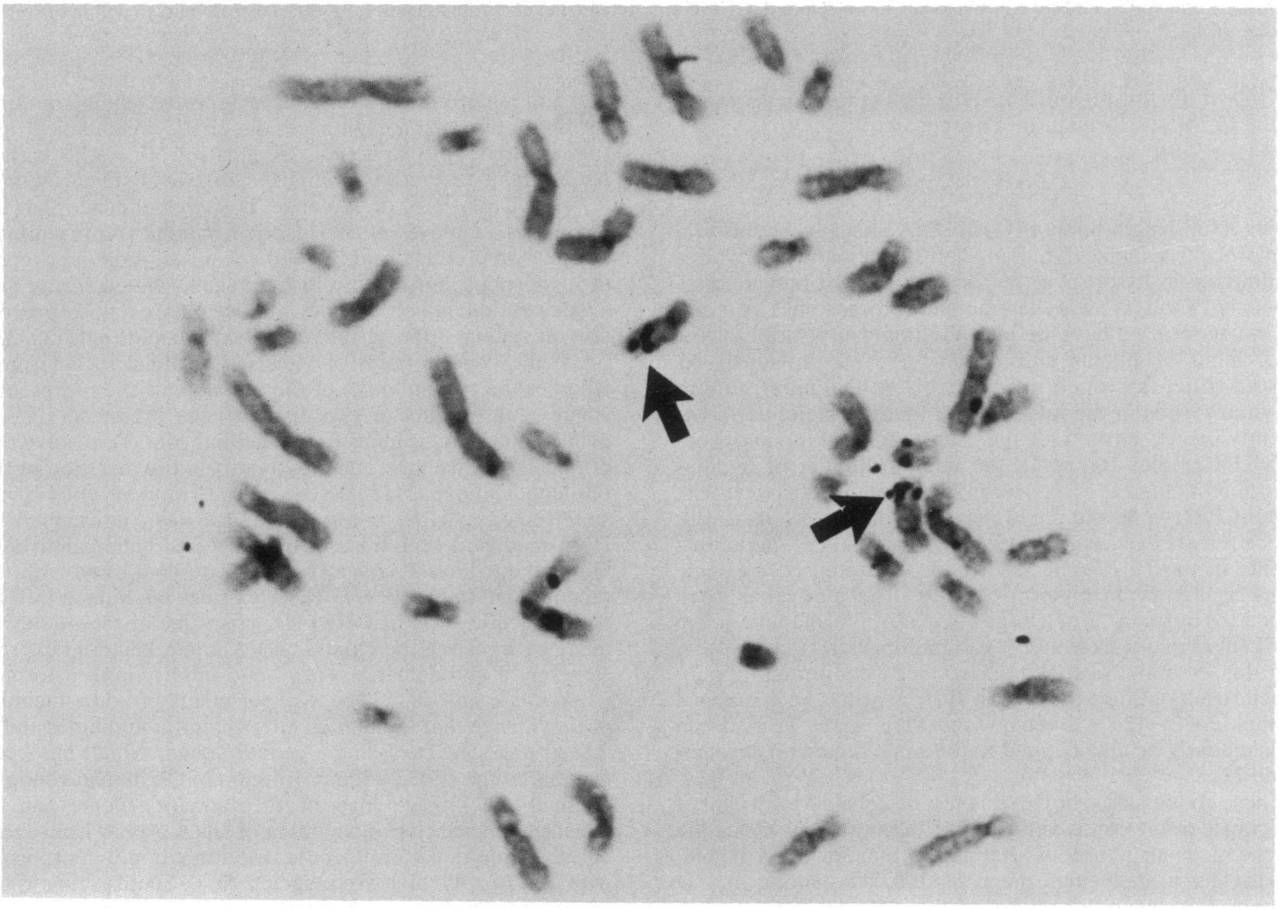

FIG. 2. Metaphase chromosome spread after *in situ* hybridization with ^{125}I-labeled phPAH247, a 2.5-kb *PAH* cDNA cloned in pBR322. The arrows point to the radioautographic grains on chromosome 12.

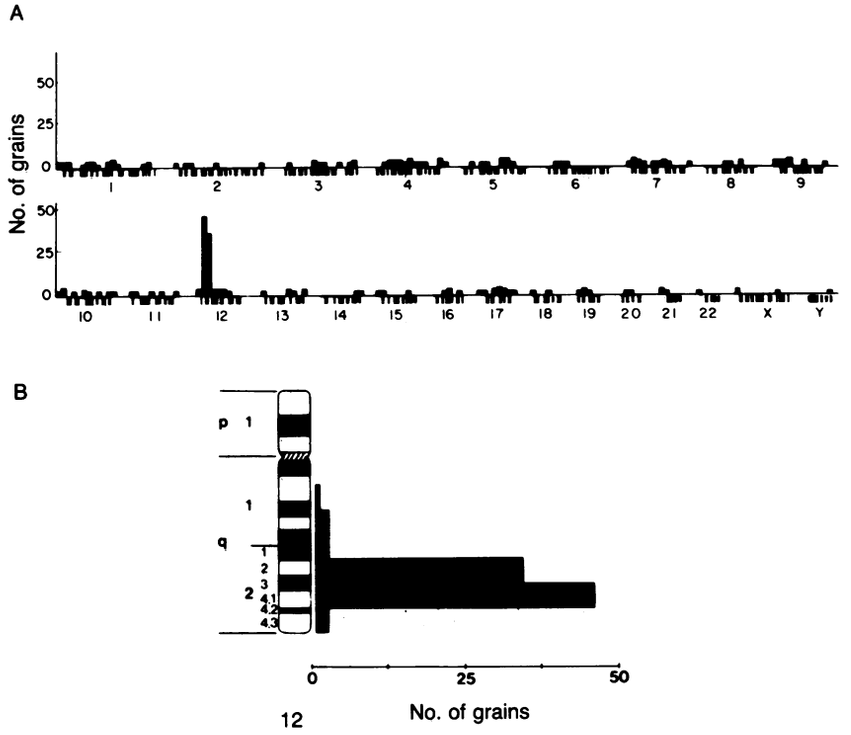

FIG. 3. Histograms for the distribution of autoradiographic silver grains. (*A*) Assignment of the human *PAH* gene to chromosome 12. After *in situ* hybridization with ^{125}I-labeled phPAH247, the distribution of grains detected over 100 chromosome spreads was plotted on an idiogram of the haploid human karyotype divided into units scaled to the average measured diameter of a silver grain (0.35 μm). Of 243 grains associated with chromosomes, 90 (37%) were assigned to chromosome 12. (*B*) Subchromosomal localization of the *PAH* gene. The distribution of silver grains observed on 37 labeled chromosomes 12 was plotted. Of 90 grains detected over chromosome 12, 81 (90%) were localized within the region 12q22→q24.1.

10^{-110}) within this region. Thus, the human *PAH* gene resides in the q22→q24.1 region of chromosome 12.

DISCUSSION

Using a full-length human *PAH* cDNA clone as a hybridization probe to analyze (*i*) human–hamster somatic cell hybrids containing an assortment of partially deleted human chromosome 12 and (*ii*) metaphase human chromosomes by *in situ* hybridization, we have defined the regional position of the *PAH* locus on human chromosome 12. We previously reported the existence of restriction fragment length polymorphisms in the human *PAH* locus, which has subsequently been used to trace the transmission of mutant genes in PKU families (6, 7). In the limited number of families reported, complete segregation concordance between the mutant *PAH* gene and the disease phenotype was observed. These results suggested that PKU is the result of mutational events in the *PAH* gene itself and is not caused by some *trans*-regulatory mechanisms (8, 9). The study has been expanded by analysis of a large number of PKU families from different ethnic backgrounds with multiple affected children, and this essential observation has been confirmed (unpublished results). Since the human *PAH* gene has been mapped to the 12q22→12q24.1 region, the PKU locus in man can consequently be also defined to the same region on chromosome 12.

These conclusions, however, must be considered in light of the considerable controversy in the literature concerning the biochemical structure of the native mammalian enzyme, which has a molecular mass of 100,000 daltons and is composed of two polypeptides of about 50,000 daltons each. Two distinct bands representing the monomeric subunits can be separated on sodium dodecyl sulfate/polyacrylamide gels, and a number of laboratories have reported finding multiple forms of the enzyme with characteristic biochemical and immunological properties, suggesting that the native heterodimer enzyme is composed of two distinct subunits (25–31). Other lines of evidence, however, indicate that the enzyme is composed of two identical subunits representing a single gene product, and the two species of monomer represent the phosphorylated and dephosphorylated forms of a single peptide, which differ in activity, charge, and apparent size on sodium dodecyl sulfate/polyacrylamide gels (32, 33). From the genetic point of view it is critical to determine whether the two subunits of the human enzyme represent a single gene product or two distinct gene products, since it could mean a single locus or two genetic loci. To resolve this critical issue, we have recently reported the insertion of the full-length human *PAH* cDNA clone into a eukaryotic expression vector and the recombinant has been introduced by DNA-mediated gene transfer into cultured mammalian cells that lack intrinsic *PAH* activity. Cells transformed with the recombinant human *PAH* cDNA express the human mRNA and authentic *PAH* activity (34). The same observation was made by inserting the cloned cDNA into a bacterial expression vector and detecting enzymatic activity in transformed *Escherichia coli* cells. These experiments provide unequivocal evidence that the human enzyme is a homodimer and is indeed encoded by a single genetic locus, which has been mapped to the 12q22→12q24.1 region in the human genome.

Finally, a linkage map of the human genome can be constructed by isolating a number of DNA probes from every chromosome that can detect restriction site polymorphisms with reasonably high frequencies (35). Such polymorphic probes can be used as linked genetic markers to analyze genetic disorders with unidentified biochemical lesions such as Duchenne muscular dystrophy on the short arm of the X

chromosome (36) and Huntington disease on the short arm of chromosome 4 (37). We have recently reported the detection of extensive restriction site polymorphisms in the human *PAH* locus with an observed heterozygosity of about 90% in the Caucasian population (38). Since the human *PAH* locus has been mapped to 12q22→12q24.1, the human *PAH* cDNA clone can also serve as a highly polymorphic marker for linkage analysis with other human gene loci. Indeed, such analysis with several random DNA probes on chromosome 12 had established linkage of one such probe with the *PAH* locus (39). The defined map position of the human *PAH* locus would thus serve as a reference point for the establishment of a detailed linkage map of human chromosome 12.

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