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# Hyperphenylalaninemia with High Levels of 7-Biopterin is Associated with Mutations in the *PCBD* Gene Encoding the Bifunctional Protein Pterin-4a-Carbinolamine Dehydratase and Transcriptional Coactivator (DCoH)

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## Summary

**Pterin-4a-carbinolamine dehydratase (PCD) is required for efficient tetrahydrobiopterin regeneration after phenylalanine hydroxylase activity. This catalytic function was proposed to be specifically defective in newborns with a mild form of hyperphenylalaninemia (HPA) and persistent high urinary levels of primapterin (7-biopterin). A second regulatory task of the same protein is DCoH, a coactivation of transcription by hepatocyte nuclear factor 1$\alpha$ (HNF-1$\alpha$), a function that is apparently not impaired in these HPA individuals. It has been shown elsewhere that the human PCD/DCoH bifunctional protein is encoded by a single 4-exon–containing gene, *PCBD*, located on chromosome 10q22. We have now examined the *PCBD* gene for mutations at the genomic level in six such HPA patients from four different families. By the use of new intron-specific primers, we detected, in all six patients, single, homozygous nucleotide alterations, in exon 4, that were inherited from their parents. These homozygous alterations predicted mutant PCD/DCoH with a single amino acid exchange, in two cases (alleles T78I), or premature stop codons, in the other four patients (alleles E86X and Q97X). Recombinant expression in *Escherichia coli* revealed that the mutant proteins—T78I, E86X, and Q97X—are almost entirely in the insoluble fraction, in contrast to wild type, which is expressed as a soluble protein. These data support the proposal that HPA in combination with urinary primapterin may be due to autosomal recessive inheritance of mutations in the *PCBD* gene specifically affecting the dehydratase activity.**

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## Introduction

Hyperphenylalaninemias (HPAs) are routinely diagnosed by the elevated plasma phenylalanine (Phe) concentration but need subsequent urinary pterin analysis, in newborns, to differentiate between various types of underlying molecular defects. The variants of HPA in which Phe hydroxylase per se is not affected are generally more severe, since they all originate from a defect in the synthesis or regeneration of tetrahydrobiopterin ($BH_4$), the obligatory Phe hydroxylase cofactor. $BH_4$ is furthermore required for the first step of dopamine and serotonin neurotransmitter biosyntheses carried out by tyrosine hydroxylase and tryptophan hydroxylase (Scriver et al. 1994, 1995). The three aromatic amino acid hydroxylases need the reduced cofactor to activate molecular oxygen and hydroxylate the corresponding amino acids (Kaufman 1993). During this enzymatic reaction, 4a-hydroxy-tetrahydrobiopterin is released and $BH_4$ is regenerated, in two enzymatic steps. The first step is dehydration to quinonoid-dihydrobiopterin by pterin-4a-carbinolamine dehydratase (PCD) (Lazarus et al. 1983). In the next step, dihydropteridine reductase recycles the cofactor to $BH_4$ in an NADH-dependent reaction (Whiteley et al. 1993). The reduced cofactor is endogenously supplied by de novo biosynthesis from GTP catalyzed by the enzymes GTP cyclohydrolase I, 6-pyruvoyl-tetrahydropterin synthase (PTPS), and sepiapterin reductase. Sufficient $BH_4$ cofactor regeneration *and* de novo biosynthesis appear to be essential for controlling plasma Phe concentration and monoamine neurotransmitter production.

The known defects in $BH_4$ metabolism include lesions in two biosynthetic enzymes, GTP cyclohydrolase I and PTPS, and in at least one cofactor-regenerating enzyme, dihydropteridine reductase (Blau et al. 1993). Autosomal recessive mutations in all the corresponding genes have been identified and correlate with persistent HPA and, at least for the severe types of defects, with neu-

rotransmitter deficiency (Smooker and Cotton 1995; Thöny and Blau 1997).

More recently, a new and apparently rare form of *transient* HPA was described with plasma Phe concentrations ranging from very high to moderately elevated (Blaskovics and Giudici 1988; Dhondt et al. 1988). Pterin analysis of this type of HPA is characterized by persistently elevated levels of urinary 7-biopterin (primapterin; Blau et al. 1989; Adler et al. 1992). This pterin derivative was shown to arise, at least under in vitro conditions, from nonenzymatic rearrangement of 4a-hydroxy-tetrahydropterin in the absence of PCD (Curtius et al. 1990; Davis et al. 1991). In agreement with this proposal, a patient was described who carried compound heterozygous mutations in the *PCBD* gene, encoding the corresponding mutant PCD proteins with reduced dehydratase activity (Citron et al. 1993; Johnen et al. 1995; Köster et al. 1995*b*). The conclusion that rapid dehydration by the homotetrameric PCD in the $BH_4$ recycling pathway is essential to prevent formation of primapterin, under a Phe load, substantiated the proposal that this new form of HPA might be due to reduced dehydratase activity (Bailey et al. 1993; Köster et al. 1995*b*, 1996; Rebrin et al. 1995).

Although the proposal that PCD activity is directly affected in such HPA patients appears rational, it is confounded by the recognition that the same "dehydratase" polypeptide has a second function, that of DCoH (Mendel et al. 1991; Citron et al. 1992; Hansen and Crabtree 1993; Hauer et al. 1993; Thöny et al. 1993). DCoH forms a heterotetrameric complex with the nuclear transcription factor hepatocyte nuclear factor 1$\alpha$ (HNF-1$\alpha$) and thereby stimulates HNF-1$\alpha$–dependent gene expression. However, there is no evidence for any diminished activity of HNF-1$\alpha$–dependent gene expression in primapterinuric patients. Moreover, direct in vivo data are not available thus far, either for reduced enzymatic PCD activity in HPA patients or for the significance of transcriptional coactivation by DCoH.

A further complication arises from the proposal that the observed reduction of PCD/DCoH dehydratase activity and subsequent accumulation of primapterin is responsible for depigmentation in melanocytes of patients with the skin disorder vitiligo (Schallreuter et al. 1994*a*, 1994*b*). In contrast to the skin depigmentation, such vitiligous patients have not been observed to suffer from HPA.

The gene for this multifunctional protein, *PCBD*, is located on human chromosome 10q22 and is expressed from a single, 4-exon–containing transcript, from which the 103–amino-acid active PCD/DCoH is encoded by exons 2–4 only (Citron et al. 1993; Hauer et al. 1993; Thöny et al. 1994*a*, 1995). The knowledge of the exact intron-exon structure has allowed us to examine the coding region for PCD/DCoH for mutations. To further test the proposal that primapterinuric patients have a genetically based defect in their PCD, we analyzed six individuals with HPA and high urinary primapterin, from four different families. The results presented here show that there are functional alterations in the corresponding gene from all patients. The association of HPA and urinary primapterin with mutations in the *PCBD* gene in all such patients corroborates the hypothesis that they are specifically defective in dehydratase.

## Patients and Methods

### *Case Reports*

Patients reported herein are registered in the BIODEF database (Blau et al. 1997). All are from nonconsanguineous parents and were identified on the basis of their high plasma Phe in neonatal screening tests.

Patient K.A. (BIODEF 264) is the first child of Caucasian parents. The initial Guthrie test, performed at age 3 d, showed a plasma Phe level of 290 $\mu$mol/liter; the recall specimen showed a level of 853 $\mu$mol/liter at 6 d and 1,997 $\mu$mol/liter at 13 d. Introduction of Lofenalac normalized the plasma Phe levels to 157 $\mu$mol/liter after 7 d. Initial screening for urinary pterins indicated a high neopterin/biopterin ratio and increased levels of primapterin (table 1). A repeat specimen, obtained at age 1 mo, showed a pattern of neopterin and biopterin being essentially normal but with persistent primapterinuria. Neurological examination of patient K.A. at age 6 d identified no abnormalities. A second evaluation at age 1 mo showed mild hypotonia of the extremities and trunk. At age 2.5 years, the Phe diet was stopped, and patient K.A. subsequently developed completely normally.

Patient J.S. (BIODEF 211) is the second child of Hispanic parents. Primapterinuria was diagnosed at age 8 d through a neonatal screening program (Blaskovics and Giudici 1988) (table 1). The patient's plasma Phe levels increased from 399 $\mu$mol/liter at age 1 d to 756 $\mu$mol/liter at age 28 d. An oral $BH_4$–loading test lowered the plasma Phe concentrations to normal levels. Following oral $BH_4$ administration (2 mg/kg/d), neopterin normalized, and biopterin as well as primapterin increased about eight-fold (Blau et al. 1989). A similar effect was obtained after administration of dihydrobiopterin (6 mg/kg) and sepiapterin (3 mg/kg) (Blau et al. 1992). The restriction of Phe intake was replaced by administration of $BH_4$ (2 mg/kg/d) for the first 8 mo of life. The only clinical manifestations of neurological impairment before introduction of $BH_4$ were transient abnormality on electroencephalogram and progressive hypotonia with a delay in motor development. Hyperphenylalaninemia

**Table 1**

**HPLC Analysis of Urinary Pterins**

| Patient | Age | Neopterin (mmol/mol creatinine) | Biopterin (mmol/mol creatinine) | Primapterin (mmol/mol creatinine) | %Biopterin[a] | %Primapterin[b] |
|---|---|---|---|---|---|---|
| K.A. | 18 d | 10.90 | .48 | .22 | 4.2 | 31.4 |
| | 1 mo | 1.10 | .80 | .14 | 42.1 | 14.9 |
| J.S. | 8 d | 16.74 | .41 | .39 | 2.4 | 48.8 |
| | 1 mo | 7.84 | .38 | .30 | 4.6 | 44.1 |
| | 7 mo[c] | 3.32 | 2.18 | 2.59 | 39.6 | 54.3 |
| | 3 years | 4.23 | 1.04 | .90 | 19.7 | 46.4 |
| T.S. | 1.5 years | 1.57 | .60 | .51 | 27.6 | 45.9 |
| | 4 years | 3.20 | 1.12 | .59 | 25.9 | 34.5 |
| | 4 years[d] | 2.41 | 2.89 | 1.34 | 54.5 | 31.7 |
| I.B. | 10 mo | 1.90 | 4.80 | 1.60 | 71.6 | 25.0 |
| | 14 mo | 1.40 | .63 | .77 | 31.0 | 55.0 |
| J.B. | 2 years | 1.20 | 1.90 | 2.30 | 61.3 | 54.8 |
| B.E. | 3 mo | 3.70 | .37 | 1.09 | 9.1 | 74.7 |
| Controls | <4 years | 1.1–4.0 | .5–3.0 | <.02 | 44–77 | NA |

[a] %Biopterin = 100 × biopterin/(neopterin + biopterin).
[b] %Primapterin = 100 × primapterin/(primapterin + biopterin). NA = not applicable.
[c] On $BH_4$ (2.5 mg/kg/d).
[d] After dihydrobiopterin loading (100 mg).

was transient, although the pterin pattern remained constant with increasing age (table 1). Patient J.S. is now 8.5 years old and, on an ordinary diet, is developing normally. A sibling, patient T.S. (BIODEF 212), who is 1.5 years older than patient J.S., showed a similar urinary pterin pattern (table 1), had a transient plasma Phe elevation in the neonatal period, and is now clinically normal (Giudici et al. 1991).

Patient I.B. (BIODEF 273) was born at term to parents of Ashkenazi Jewish origin. Newborn screening at age 3 d showed plasma Phe at 240 $\mu$mol/liter. Repeated measurements on day 14 showed plasma Phe levels of 1,845 $\mu$mol/liter. On a low-Phe diet (38 mg/kg/d), the patient's plasma Phe level fell rapidly to a normal level over a period of 36 h. Patient I.B. has a high tolerance for Phe of ~80–90 mg (Arn et al. 1994). The patient's pterin profile in urine at age 2 wk showed 1.8% of biopterin (E. Naylor, personal communication), suggesting PTPS deficiency. Dihydropteridine reductase activity was normal. Repeated plasma and urine measurement at age 3 wk showed, again, an elevated neopterin-to-biopterin ratio. An oral $BH_4$–loading test (10 mg/kg body weight) resulted in a decrease of plasma Phe from 540 $\mu$mol/liter to 234 $\mu$mol/liter and then to 196 $\mu$mol/liter within 4 and 8 h, respectively. At age 1 mo, pterin and neurotransmitters in the cerebrospinal fluid (CSF) were normal, suggesting a peripheral biopterin deficiency (K. Hyland, personal communication). The patient was put on a Phe-restricted diet, together with administration of $BH_4$ (5 mg/kg/d).

We reevaluated patient I.B. at age 2 mo and found a normal PTPS activity in the red blood cells of the patient; of his sister, patient J.B. (BIODEF 344); and of both parents. The urinary pterin profile, during $BH_4$ treatment, showed normal neopterin and biopterin but increased primapterin (table 1), indicating a deficiency of PCD. The patient was treated with an unrestricted diet and oral $BH_4$ (10 mg/kg/d) for 4 mo. After 4 mo, $BH_4$ was discontinued, and Phe levels, which were 120–300 $\mu$mol/liter, are still within this range at age 5 years. Both patient I.B. and his sister, patient J.B., who has the same genotype, are developing normally. Neither child is on pharmacological treatment or a restricted diet. Hyperphenylalaninemia was never documented in the 18-mo-older sibling.

Patient B.E. (BIODEF 272) is the second child of unrelated parents. Pregnancy and delivery were normal. Birth weight was 2.9 kg. At the initial neonatal screen at age 3 d, plasma Phe was 183 $\mu$mol/liter. This is below the "normal" cut-off of 240 $\mu$mol/liter, so instead of the usual follow-up, a repeat neonatal screen was done at 3 wk, at which time plasma Phe was found to be 1,600 $\mu$mol/liter. The child was immediately brought to the clinic center. The peak Phe level, at 3.5 wk, was 2,589 $\mu$mol/liter. The child was started on a low-Phe formula, and levels were stabilized, mostly <400 $\mu$mol/liter. He was observed to have considerable Phe tolerance (~100 mg/kg). The initial urinary pterin profile (F. Moyhuddin, personal communication) showed an increased neopterin-to-biopterin ratio of 15, indicating a defect in the biosynthesis of $BH_4$. A $BH_4$-loading test (6 mg/kg) at age 6 wk showed only a slight response (Phe at 0 h, 731

μmol/liter; at 3 h, 612 μmol/liter; and at 6 h, 502 μmol/liter). A week later, at a $BH_4$ dose of 22 mg/kg, there was a definite response (Phe at 0 h, 773 μmol/liter; at 3 h, 517 μmol/liter; and at 6 h, 155 μmol/liter). CSF neurotransmitter concentrations were normal, suggesting a peripheral deficiency (K. Hyland, personal communication). Reevaluation at age 3 mo established the diagnosis of primapterinuria. We found normal PTPS activity in the red blood cells of patient B.E., of his sister, and of both parents. Percentage of urinary biopterin was rather low, and increased concentrations of primapterin were found (table 1). He was initially treated with 25 mg $BH_4$ daily. This was discontinued, at age 4 mo, with no change in Phe levels; since then, Phe levels have remained stable, at 100–250 μmol/liter, on an unrestricted normal diet. Clinical assessment at age 3.5 years demonstrated normal growth and development. The patient was noted to have prominent epicanthic folds and increased carrying angles at the elbows but no other dysmorphic features.

### HPLC Analysis of Urinary Pterins and CSF Metabolites

$BH_4$ was purchased from Dr. Schircks's laboratory in Jona, Switzerland. HPLC of pterins, after oxidation with manganese dioxide, was performed as described elsewhere (Curtius et al. 1991).

### Genomic DNA Analysis by Exon-Specific Amplification and Direct Sequencing of PCR Products

Genomic DNA was isolated from blood leukocytes by means of standard extraction procedures (Sambrook et al. 1989). Oligonucleotides for PCR and for sequencing were synthesized on a Gene Assembler Plus DNA synthesizer (Pharmacia Biotech) and are compiled in table 2. Conditions for PCR amplification were as follows: 1 μg genomic DNA was incubated in a 40-μl PCR reaction containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 200 mM dNTP, 40 pmol of each primer, 0.8 U *Taq* polymerase (Promega), and either 1.5 mM $MgCl_2$, for exon 2, or 3 mM $MgCl_2$, for exons 3 and 4. Amplification was carried out in a Perkin-Elmer Cetus DNA thermocycler for 35 cycles, with denaturation at 95°C for 1 min, annealing at 62°C for 1 min, and extension at 72°C for 1 min. A final extension step at 72°C for 7 min was added. PCR products were separated on a 1% agarose gel and purified by use of a Qiagen Gel Extraction Kit. Sequencing was performed in accordance with a procedure described elsewhere (Thöny et al. 1994*b*).

**Table 2**

**Oligonucleotides for *PCBD*-Exon Amplifications and for DNA Sequence Analysis Using Intronic Primers**

| Exon | Sense Primer | Antisense Primer | Product Size (bp) | Sequencing Primer |
|---|---|---|---|---|
| 2 | PCDH60 | PCDH23 | 413 | PCDH60 |
| 3 | PCDH62 | PCDH61 | 538 | PCDH73 |
| 4 | PCDH64 | PCDH63 | 721 | PCDH74 |

NOTE.—Primer sequences: PCDH23: 5′-(7637) AAG ACA CAT CCT CCT ATG GC (7618)-3′; PCDH60: 5′-(7225) TGG AGC CCC TCT GTT TTG AC (7244)-3′; PCDH61: 5′-(8246) AGG GGT ATT TAT GTT GAT TC (8227)-3′; PCDH62: 5′-(7709) TGA AGA CAT TTG AAC AGA CG (7728)-3′; PCDH63: 5′-(9720) GAG CCT GAG ACC AAG TGA TA (9701)-3′; PCDH64: 5′-(9000) AGG GGA GAT GTA GAA GAT GT (9019)-3′; PCDH73: 5′-(7899) TTC ATC CTC CTG CGT CTA (7916)-3′; PCDH74: 5′-(9112) GTG ACT CCC TCC TGT TCT T (9130)-3′. The nucleotide numbers given in parentheses refer to the published *PCBD* gene sequence (GenBank accession number L41560; Thöny et al. 1995).

### Recombinant Expression of Mutant PCD/DCoH

An *Escherichia coli* plasmid vector expressing the T87I-PCD/DCoH recombinant mutant protein was generated by two consecutive PCR amplifications, basically as described elsewhere, using the 5′ and 3′ primers PCDH15 (5′-CGG AAT TCA TAT GGC TGG CAA AGC ACA CAG-3′) and PCDH16 (5′-CGG GAT CCT ATG TCA TGG ACA CTG CTA C-3′) and the mutagenic primer PCDH80 (5′-TCA CGC TGA GCA TCC ATG AGT GTG C-3′) (Köster et al. 1995*a*, 1995*b*). The PCDH15 primer contains two consecutive recognition sequences for the enzymes *Eco*RI and *Nde*I, and primer PCDH16 harbors a *Bam*HI site. After amplification and restriction-enzyme digestion, the *Eco*RI/*Bam*HI fragment was subcloned into pUC18 (plasmid pHDH65). Subsequently, the *Nde*I/*Bam*HI fragment was isolated from pHDH65 and inserted into pGEMEX-2-Nde to yield plasmid pHDH66. Plasmid constructs for the truncated mutants E86X- and Q97X-PCD/DCoH were generated in a similar way. Fragments were directly amplified from the wild-type cDNA template by use of the 5′ primer PCDH15 in combination with either PCDH76 (5′-CGG GAT CCT TAT GAA AGG CCG GCA CAC TCT A-3′), for mutant E86X, or PCDH77 (5′-CGG GAT CCT TAT TCG ATG AAG CTG GCC AGG T-3′), for mutant Q97X. This yielded the pUC18 derivatives pHDH60 (E86X) and pHDH61 (Q97X) and the corresponding pGEMEX-2-Nde plasmid derivatives pHDH63, for mutant E86X-PCD, and pHDH64, for Q97X-PCD. Expression was achieved from the p-GEMEX-2-Nde derivatives by standard isopropyl β-D-thio-galactopyranoside induction, for 3 h, of logarithmically growing cultures of *E. coli* BL21(DE3) (Promega). Cells were harvested by centrifugation, resuspended in 5 mM Tris-HCl, pH 8.0, 40 ml/liter of cells, and fast frozen.

**Table 3**

**Mutation Analysis of the *PCBD* Gene from Six HPA Patients with 7-Biopterins**

| Family and/or Individual (Status) | Base-Pair Change[a] | | | Amino Acid Change[b] |
|---|---|---|---|---|
| | Exon 2 | Exon 3 | Exon 4 | |
| K.A. (male, patient) | wt | wt | 312C→T/312C→T | Q97X homozygous |
| Family S: | | | | |
| H.S. (mother) | wt | wt | 256C→T/wt | T78I heterozygous |
| J.S. (daughter, patient) | wt | wt | 256C→T/256C→T | T78I homozygous |
| T.S. (son, patient) | wt | wt | 256C→T/256C→T | T78I homozygous |
| Family B: | | | | |
| S.B. (father) | wt | wt | 279G→T/wt | E86X heterozygous |
| A.B. (mother) | wt | wt | 279G→T/wt | E86X heterozygous |
| I.B. (son, patient) | wt | wt | 279G→T/279G→T | E86X homozygous |
| J.B. (daughter, patient) | wt | wt | 279G→T/279G→T | E86X homozygous |
| Family E: | | | | |
| R.E. (father) | wt | wt | 312C→T/wt | Q97X heterozygous |
| D.C. (mother) | wt | wt | 312C→T/wt | Q97X heterozygous |
| B.E. (son, patient) | wt | wt | 312C→T/312C→T | Q97X homozygous |

[a] wt = wild-type sequence.

[b] X = termination codon. Since the N-terminal methionine is cleaved from the recombinant dehydratase as well as from the enzyme isolated from human and rat liver, methionine is not included in the numbering of the amino acid residues (Hauer et al. 1993; Köster et al. 1995*a;* Ayling et al. 1997).

### *Extraction of Mutant Dehydratases*

Cells containing mutant dehydratases were lysed either by sonication or by freeze/thawing. In the first method, the cell paste was thawed and sonicated, in 5-ml aliquots, with a Branson Sonifier 250 set at 6. Sonication was carried out on ice, for three cycles of 15 s each, with 1 min cooling between each cycle. In the second method, the cell paste was thawed, rapidly refrozen in liquid nitrogen, and quickly thawed twice more. The latter method of extraction was found to be advantageous for measuring low levels of dehydratase activity in crude extracts. The lysates were centrifuged at 100,000 *g* for 60 min at 4°C, and the supernatants were frozen in liquid nitrogen and stored at −80°C. In all cases, samples of the lysates were removed before centrifugation and were saved for SDS-PAGE analysis. SDS-PAGE was performed by means of a procedure optimized for the separation of low–molecular-weight proteins (Schägger and von Jagow 1987), with 10% acrylamide in the separating gel.

### *Dehydratase-Activity Assay*

Enzyme activity was measured by use of chemically synthesized 4a-hydroxy-tetrahydropterins (Bailey et al. 1993, 1995), as described elsewhere (Bailey et al. 1993; Rebrin et al. 1995). Activities were measured at 10°C in 10 mM Tris-HCl, pH 8.2, in the presence of an excess of dihydropteridine reductase (5 units/ml reaction) and NADH (0.1 mM), with 6(S)-propyl-4a-hydroxy-tetrahydropterin (20 $\mu$M) as substrate. At this temperature, buffer concentration, and pH, nonenzymatic dehydration of substrate is minimized. Reactions were monitored at 340 nm until completed, and data were acquired by computer. The first-order rate constant for nonenzymatic dehydration was measured separately and entered as a constant in fitting data to the rate equation. Maximum velocities and Michaelis constants were calculated from the rate equation for the complete progress curve by use of the PC-compatible nonlinear regression program "Scientist" (Micromath), as detailed elsewhere (Rebrin et al. 1995).

## Results

### *Mutations in the* PCBD *Gene from HPA Patients with Primapterinuria*

The mature 103-residue PCD/DCoH protein monomer is encoded by exons 2–4 of the human *PCBD* gene (Citron et al. 1993; Thöny et al. 1995). We thus established PCR conditions to amplify these three exons from genomic DNA from patients (table 2), and we examined the coding sequences and flanking splice sites by direct nucleotide-sequence determination. We analyzed six HPA patients with primapterinuria, including family members when available (table 3). Whereas no mutations were detected in exons 2 and 3, all patients exhibited, homozygously, single-nucleotide alterations in exon 4 that originated from the corresponding parents.

Moreover, the mutations predicted an amino acid exchange or premature termination codons (see fig. 1). A mutant allele, Q97X, an exchange from Gln97 to a termination codon, was detected in patient K.A. (fig. 1*A*) and, in family E, in patient B.E. (fig. 1*D*). In family S, patients T.S. and J.S. had a mutation predicting a homozygous Thr78→Ile alteration (allele T78I; fig. 1*B*). The two patients I.B. and J.B., from family B, had an exchange from Glu86 to a termination codon (allele E86X; fig. 1*C*).

### *Expression of Mutant Dehydratases*

To investigate the effect of alterations in sequence on protein function, we expressed these mutant dehydratases in *E. coli.* In all cases, the wild-type recombinant dehydratase, which has properties identical to those of the human liver enzyme (Ayling et al. 1997), was run as a control. Analysis by SDS-PAGE showed that all of the wild-type dehydratase is in the soluble fraction (100,000 *g* supernatant), where typically one-third of the soluble protein is dehydratase. SDS-PAGE analysis of the 100,000 *g* supernatants of each of the mutants revealed only negligible amounts of protein at the dehydratase position. On the other hand, when solubilized whole-cell lysates were analyzed by SDS-PAGE, the amount of mutant protein corresponding to dehydratase was similar to that obtained with wild type. Thus, it appears that, although soluble and functional wild-type dehydratase can be expressed in *E. coli,* almost all of the mutant protein is in an inactive, insoluble form.

### *Catalytic Activity of Mutant Dehydratases*

Since only a small fraction of the mutant dehydratase was in the soluble fraction, the cell extracts were analyzed for catalytic activity without further protein purification. The 100,000 *g* supernatants were concentrated in a Centricon 30 (Amicon), washed with enzyme-assay buffer, and concentrated again. Surprisingly, the extracts of the truncation mutants, Q97X and E86X, contained activity, although at low levels. In an attempt to increase the amount of these proteins in the soluble fraction, *E. coli* containing the mutant dehydratases were grown at 20°C. This resulted in a 10-fold increase in E86X and a 4-fold increase in Q97X activity in the soluble fraction, compared with the activities of the extracts from cells grown at 37°C. The Michaelis constant (*Km*) of 6-propyl-4a-hydroxy-tetrahydropterin with E86X appeared to be similar to that of wild type, whereas with Q97X, the *Km* was two to three times higher. Since the amounts of Q97X and E86X mutant proteins in the soluble fractions were insufficient to enable purification, turnover values for these mutants could not be determined. No activity could be detected in the T78I extract, which indicates either that there was insufficient dehydratase protein in the soluble fraction or that the dehydratase in the soluble fraction has very low or no activity. Attempts to obtain any T78I activity in the soluble fraction were without success, which indicates either that the dehydratase in this fraction had very low or no activity or that insufficient dehydratase protein was solubilized.

## Discussion

Primapterin has been shown to arise from rearrangement of 4a-hydroxy-$BH_4$, the substrate of PCD. It was therefore postulated that primapterinuria may be due to a defect in the dehydratase (PCD). Through analysis of the dehydratase gene of six patients with transient HPA and persistent urinary primapterin, we found that all have homozygous alterations and that the parents were heterozygous for the mutation. Therefore, this defect appears to be an autosomal recessive trait (MIM 264070). In addition to the six patients reported here, another patient with similar symptoms was found to have compound heterozygous mutations in the *PCBD* gene (Citron et al. 1993). Moreover, five more patients currently being characterized all show mutations in exons 2, 3, or 4 of the *PCBD* gene (Blau et al. 1997; Thöny et al., in press).

With the information about the site and type of codon alteration, it would be of interest to determine whether there is any correlation between genotype and phenotype. However, the severity of a mutation such as, for example, the truncated E86X allele appears not to lead to a specifically higher percentage of urinary primapterin when compared among the different patients. Moreover, the identical mutant allele Q97X homozygously present in two patients gave rise to 14.9% primapterin at age 1 mo for patient K.A. and 74.7% primapterin at age 4 mo for patient B.E. (table 1). Thus, the variability in the amount of urinary primapterin (or plasma Phe) appears, at least with the available data, not to be correlated to the observed mutations.

The functional consequences of the predicted amino acid alterations for dehydratase activity were analyzed. When wild-type human dehydratase is expressed in *E. coli,* all of the dehydratase is found in the soluble fraction, even when expression levels of the dehydratase are as high as 35% of the soluble protein. The enzyme can be extracted and purified in >90% yield and is fully active, with catalytic parameters indistinguishable from those of enzyme isolated from rat or human liver (Ayling et al. 1997). Under identical conditions of expression, essentially all of the dehydratase of the mutants T78I, E86X, and Q97X was in the insoluble fraction. However, at least with E86X and Q97X, the small amount of dehydratase in the soluble fraction was sufficient to show that both of these mutants do have some catalytic

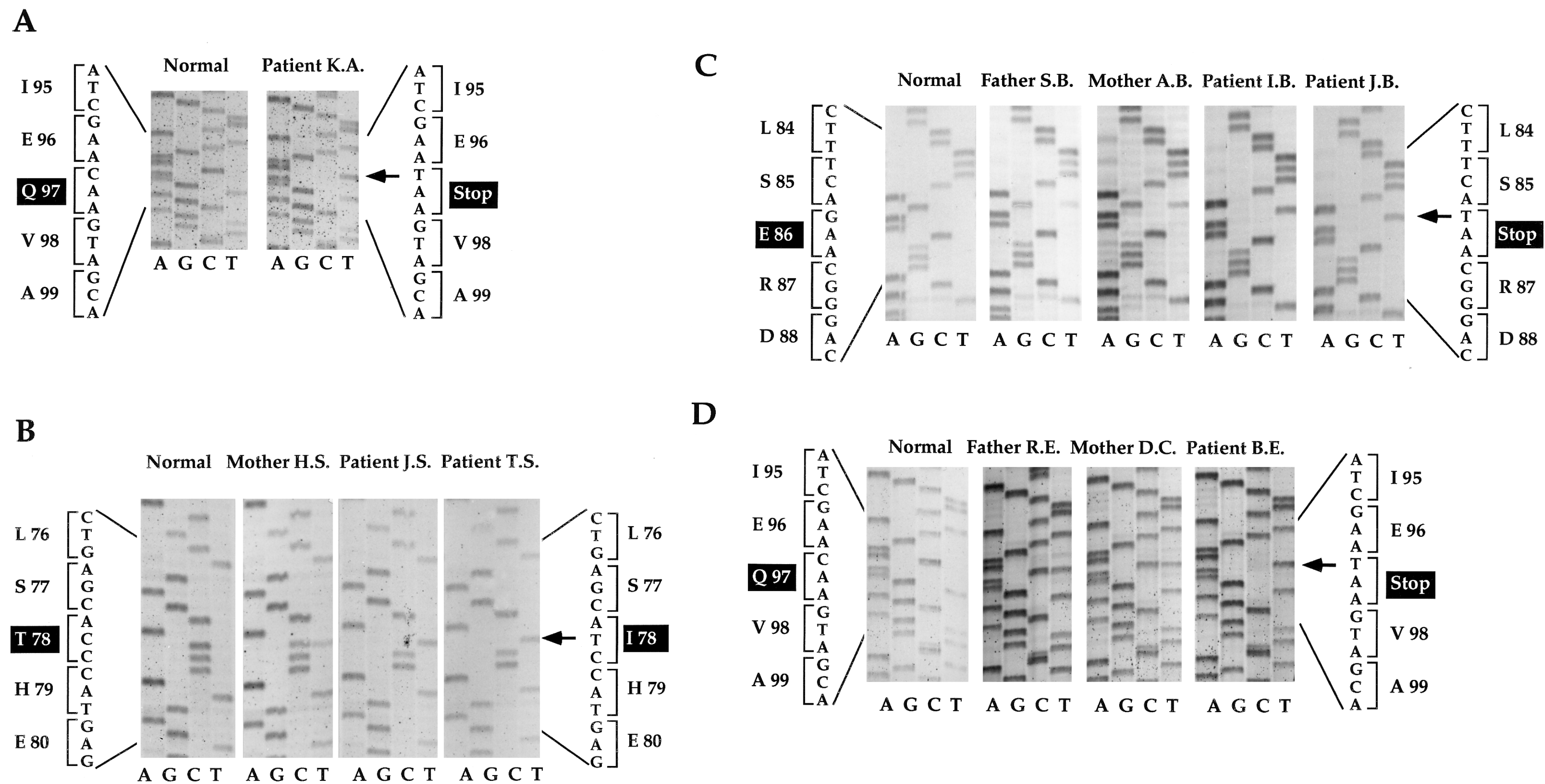


**Figure 1** Sequence analysis and detection of mutations in exon 4 of the *PCBD* gene in HPA patients. *A*, Patient K.A. *B*, Family S. *C*, Family B. *D*, Family E. Direct nucleotide-sequence determination of PCR products was performed as described in Patients and Methods. Arrowheads mark the site of base alterations detected. The coding triplets and corresponding amino acids, numbered according to the PCD/DCoH-cDNA, are indicated (Thöny et al. 1995).

activity and that both retain the ability to bind substrate with a *Km,* which does not differ significantly from wild type. The E86X truncated mutant even lacks the complete C-terminal $\alpha$3-helix. Thus, although the $\alpha$3-helix contributes to catalytic activity, it is not absolutely essential. Whether the inability to detect any catalytic activity with T78I is due to its being totally insoluble, or totally inactive, will require expression in another system with less propensity to package recombinant proteins into insoluble forms. A recombinant system that retains a higher percentage of the expressed protein in the soluble fraction will also allow E86X and Q97X to be purified and the specific catalytic activities of these two mutants to be compared to the catalytic activity of wild-type dehydratase.

The observation that each of the three mutant dehydratases is found primarily in the insoluble fraction when expressed in *E. coli*, even though all the wild type is in the soluble fraction, reveals that the mutations have a significant effect on the folding of the protein. This suggests that, in vivo, these proteins may not fold correctly or may fold sufficiently slowly that they are susceptible to proteolysis.

All these data together support the hypothesis that this benign form of HPA with high urinary levels of primapterin is caused by recessive mutations in the pterin-4a-carbinolamine dehydratase gene. Although the *PCBD* mutations may result in mild HPA and excretion of high levels of primapterin, no symptoms indicating defective DCoH function have been observed so far. Studies of the binding of dehydratase/DCoH to HNF-1$\alpha$ and the effect on its transcriptional activity have been performed, in vitro and in vivo, with several synthetic mutants of DCoH (Johnen and Kaufman 1997; Sourdive et al. 1997). So far, these studies have revealed that enzymatic activity and binding to HNF-1$\alpha$ are not necessarily correlated. Nevertheless, the mutations found in the patients reported here that may render the dehydratase more susceptible to incorrect folding or to proteolysis have yet to be investigated for their effects on the proposed DCoH function. The binding of these mutant PCDs to HNF-1$\alpha$ and the consequences for HNF-1$\alpha$ transcriptional activation are topics for future investigation.

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## Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

BIODEF database, http://www.unizh.ch/~blau/biodef1.html (for patients K.A. [264], J.S. [211], T.S. [212], I.B. [273], J.B. [344], and B.E. [272])

GenBank, http://www.ncbi.nlm.nih.gov/Web/Genbank (for *PCBD* gene [L41560])

Online Mendelian inheritance in man (OMIM), http://www.ncbi.nlm.nih.gov/htbin-post/Omim (for primapterinuria [MIM 264070])

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