

Single-Step Mutation Scanning of the 6-Pyruvoyltetrahydropterin Synthase Gene in Patients with Hyperphenylalaninemia

ANNE ROMSTAD,¹ PER GULDBERG,¹ NENAD BLAU,² and FLEMMING GÜTTLER^{1*}

Background: Deficiency of 6-pyruvoyltetrahydropterin synthase (PTPS) is a recessively inherited disorder that leads to depletion of 5,6,7,8-tetrahydrobiopterin, the obligatory cofactor for hydroxylation of phenylalanine, tyrosine, and tryptophan. A marker for neonatal detection of PTPS deficiency is hyperphenylalaninemia (HPA). Molecular analysis would provide a simple and reliable means for distinguishing PTPS deficiency from other potential causes of HPA.

Methods: We developed a method based on PCR in combination with denaturing gradient gel electrophoresis (DGGE) that rapidly scans the six coding sequences and all splice sites of the PTPS gene (*PTS*) for mutations. This method was used to examine the status of the *PTS* gene in control samples with known *PTS* mutations and in five patients with PTPS deficiency.

Results: Two features of the *PTS* gene posed particular problems in relation to DGGE analysis: the very high GC content of exon 1, and a 15-bp poly(dT) stretch in the acceptor splice site of intron 1. Both problems were solved by special design of amplification primers. PCR and DGGE conditions were adjusted to allow simultaneous analysis of all six regions of the *PTS* gene. Using this one-step approach, all control mutations were readily resolved. Among the five PTPS patients, four mutations were identified, including IVS1-3C→G, IVS2-7T→A, V57del, and V97M (289G→A). The IVS1-3C→G mutation was shown by reverse transcription-PCR analysis to produce multiple splice variants.

Conclusions: We have established a fast and reliable screening method for detection of mutations and small deletions/insertions in the *PTS* gene. This method

should be useful for rapid diagnosis of PTPS deficiency in newborns with HPA.

© 1999 American Association for Clinical Chemistry

Hyperphenylalaninemia (HPA)³ detected by neonatal screening programs is caused by a deficiency of either phenylalanine hydroxylase (PAH; EC 1.14.16.1), accounting for 98–99% of all HPA cases, or its essential cofactor, 5,6,7,8-tetrahydrobiopterin (BH₄) (1, 2). BH₄ serves as a cofactor for glyceryl-ether monooxygenase (EC 1.14.16.5), nitric-oxide synthase (EC 1.14.13.39), and for the two other aromatic hydroxylases, tyrosine hydroxylase (EC 1.14.16.2) and tryptophan hydroxylase (EC 1.14.16.4) (3). The two latter enzymes catalyze the rate-limiting steps in the biosynthesis of catecholamines and serotonin.

The synthesis of BH₄ is carried out by three enzymes, GTP cyclohydrolase I (EC 3.5.4.16), 6-pyruvoyltetrahydropterin synthase (PTPS; EC 4.6.1.10), and sepiapterin reductase (EC 1.1.1.153). Through the activity of, for example, PAH, BH₄ is oxidized to quinonoid dihydrobiopterin and is recycled to BH₄ by pterin-4 α -carbinolamine dehydratase (EC 4.2.1.96) and dihydropteridine reductase (EC 1.6.99.7) (2). Disorders of the BH₄ system generally are characterized by HPA and neurological symptoms attributable to lack of the neurotransmitters dopamine and serotonin. Deficiency of BH₄ can be caused by defects in either the synthesis or the recycling of BH₄. According to the International Database of Tetrahydrobiopterin Deficiencies, more than one-half (58%) of all BH₄ deficiencies are caused by defects in the PTPS enzyme (4).

Diagnostic procedures to differentiate between PAH and BH₄ deficiencies include measurement of urinary pterins or oral loading tests with BH₄ (5, 6). Prenatal

¹ The John F. Kennedy Institute, Gl. Landevej 7, 2600 Glostrup, Denmark.

² Division of Clinical Chemistry and Biochemistry, University Children's Hospital, Steinwiesstrasse 75, 8032 Zurich, Switzerland.

*Author for correspondence. Fax 45-4343-1130; e-mail flg@kennedy.dk.

Received July 26, 1999; accepted September 21, 1999.

³ Nonstandard abbreviations: HPA, hyperphenylalaninemia; PAH, phenylalanine hydroxylase; BH₄, 5,6,7,8-tetrahydrobiopterin; PTPS, 6-pyruvoyltetrahydropterin synthase; and DGGE, denaturing gradient gel electrophoresis.

diagnosis of PTPS deficiency at 15–24 weeks gestation has been made on the basis of pterin concentrations in amniotic fluid and enzymatic activity in fetal erythrocytes (7).

Clinically, PTPS deficiency is a heterogeneous disease with large variations in residual PTPS activity in erythrocytes, in concentrations of neurotransmitter metabolites (especially in cerebrospinal fluid), in the degree of HPA, and in neurological symptoms (typically hypotonia of the trunk and hypertonia of the extremities) (2). The “severe” or “typical” form of PTPS deficiency is characterized by lowered concentrations of neurotransmitter metabolites in cerebrospinal fluid and severe neurological symptoms. This form requires treatment with BH₄, neurotransmitters, and their precursors such as L-3,4-dihydroxyphenylalanine, carbidopa, and 5-hydroxytryptophan (8,9). Less severely affected patients are assigned to a heterogeneous group encompassing mild/peripheral or “atypical” forms of PTPS deficiency. The disease course in this group varies from cases with transient neonatal HPA (10) to cases where a mild form proceeds into a severe form with changes in cerebrospinal fluid neurotransmitter metabolites (11).

The gene encoding the PTPS enzyme (*PTS*) has been cloned and found to span ~8 kb and consist of six exons (12,13). The gene maps to chromosome 11q22.3-q23.3 (14) and encodes the 145 amino acids of each subunit of the homohexameric enzyme (13,15).

A method for fast identification of mutations causing PTPS deficiency would be a substantial improvement for diagnostic and therapeutic purposes, both for early determination of treatment requirements and for prenatal diagnosis. Here, we present an approach based on PCR and denaturing gradient gel electrophoresis (DGGE) to rapidly and simultaneously scan all six exons and flanking intronic sequences of *PTS* for mutations. We have evaluated the efficacy of this method by analyzing DNA with known *PTS* mutations, and we have applied the method for the detection of mutations in patients clinically diagnosed as having PTPS deficiency.

Materials and Methods

PATIENT SAMPLES

All patients were originally referred to The John F. Kennedy Institute after a positive result in the Guthrie screening test. The phenylalanine concentrations, urinary pteridine values, and PTPS enzyme activities in red blood cells (16) are summarized in Table 1. Three of the patients belonged to the group with severe PTPS deficiencies, one had a mild/transient course, and one was a putative carrier. The procedures used were in accordance with the current revision of the Helsinki Declaration of 1975.

DNA EXTRACTION

Genomic DNA was extracted from 10 mL of EDTA-anticoagulated blood by standard procedures (17). DNA samples with previously characterized mutations in the *PTS* gene were kindly provided by Drs. Beat Thöny [mutations R16C (46C→T), T67M (200C→T), P87L (260C→T) and D136V (407A→T)] and Harvey L. Levy [mutation N47D (139A→G)], and were used for optimizing the method.

PRIMER DESIGN

Optimal primers for amplification of the six exons of the *PTS* gene with surrounding intron sequences were selected using the OLIGO 4.0 Program from National Biosciences (18). Subsequently, computer simulations of the melting behaviors of the six PCR products were carried out using MELT87 (19) to enable the addition of appropriate GC clamps to one of the primers in each primer pair, thus yielding optimal melting behavior for each exon. All primers are listed in Table 2.

PCR AMPLIFICATION AND DGGE ANALYSIS

PCR amplification was carried out in 25- μ L reactions, using a commercial buffer and *Taq* polymerase (DNA *Taq* polymerase; Qiagen). All reactions contained 1.5 mmol/L MgCl₂, 200 μ mol/L dNTPs (Pharmacia Biotech), 20 pmol of each primer, 2 U of *Taq* polymerase, 200 ng of DNA,

Table 1. Biochemical values, clinical phenotypes, and status of the *PTS* gene.

| Patient | BIODEF no. ^a | Blood Phe, μ mol/L | Urinary concentration, mmol/mol creatinine | | PTPS RBC ^b activity, μ U/g Hb | Mutations | | Phenotype |
|---|-------------------------|---------------------------|---|-----------|--|---------------|-----------|---------------------------|
| | | | Neopterin | Biopterin | | Allele 1 | Allele 2 | |
| A | 16 | 1800 | 34.3 | 0.41 | 1.3 | V57del | IVS2-7T→A | Severe |
| B | 274 | 265 ^c | 4.3 | 0.19 | 0.6 | IVS1-3C→G | IVS1-3C→G | Severe |
| C | 275 | 540 ^c | 4.1 | 0.09 | 0.82 | IVS1-3C→G | IVS1-3C→G | Severe |
| D | 109 | 244 | 3.17 | 0.28 | 2.8 | IVS1-3C→G | | Mild/transient |
| E | | 300 | 3.6 | 1.0 | 3.6 | V97M (289G→A) | | Heterozygote ^d |
| Reference values for children <10 years ^e | | <120 | 1.1–4.0 | 0.5–3.0 | 34–64 | | | |

^a BIODEF numbers from the International Database on Tetrahydrobiopterin Deficiencies (4).

^b RBC, red blood cell; Hb hemoglobin.

^c On a phenylalanine-restricted diet.

^d See Results.

^e From Blau and Blaskovics (5).

Table 2. Primer sequences used in this study.

| <i>PTS</i> exon | Primer | Primer sequences (5'-3') ^a | Expected size, bp |
|-----------------|------------|--|-------------------|
| Exon 1 | 6-PTS-1A | 5'-GGCCGAGCACCCGACAGC-3' | 202 |
| | 6-PTS-1B | 5'-(+56 GC) CCCGCACGCCCGCGCTGTACC-3' | |
| Exon 2 | 6-PTS-2A | 5'-(+40 GC) GTGTCATGCTGACTTTTCTTTGTTTTG-3' | 181 |
| | 6-PTS-2B | 5'-CTGAAATGTCATCAGTTTTTC-3' | |
| Exon 3 | 6-PTS-3A | 5'-(+40 GC) TGTTGCCAAGTTGTGCTGTATG-3' | 220 |
| | 6-PTS-3B | 5'-CACCACACTGGCTGAAAGAGAG-3' | |
| Exon 4 | 6-PTS-4A | 5'-(+40 GC) CAGCCGTTAATATGGAGAGCC-3' | 228 |
| | 6-PTS-4B | 5'-GGGGAGGTAGATAGGGGGC-3' | |
| Exon 5 | 6-PTS-5A | 5'-(+40 GC) TGGAACAATTTGGAATTTGAGTC-3' | 219 |
| | 6-PTS-5B | 5'-CTTGTTTTACAATCCACATAGGC-3' | |
| Exon 6 | 6-PTS-6A | 5'-(+40 GC) TTGTTTGCATTTTGAATTTTTTTG-3' | 258 |
| | 6-PTS-6B | 5'-TCAAACACAGAAAGAACTGGGC-3' | |
| | PTS-RNA-1A | 5'-AAGGTGGTGGCCGTCGCTGC-3' | |
| | PTS-RNA-1B | 5'-TGGCCATTGGATTGTTGCATTCC-3' | |

^a Numbers in parentheses indicate the lengths of the GC-clamps. The two underlined bases in primer 6-PTS-2A are bases modified from Ts (see text). Primers PTS-RNA-1A and PTS-RNA-1B were used for reverse transcription-PCR.

and a loading buffer consisting of 0.08 g/L cresol red (Aldrich) and 120 g/L sucrose. Amplification of exon 1 required the addition of 75 mL/L dimethyl sulfoxide (Sigma). Amplification was carried out in a Perkin-Elmer Cetus Thermocycler (PE 9600) with the following cycling parameters: 5 min at 94 °C; 40 rounds of 94 °C (30 s), 55–58 °C (30 s), and 72 °C (30 s); and 7 min at 72 °C. To complete the formation of heteroduplexes, the reaction was followed by 10 min at 99 °C, a 10-min ramp to 65 °C, 50 min at 65 °C, a 10-min ramp to 37 °C, and 50 min at 37 °C.

Gels for DGGE were made between glass plates of 20 × 18 cm with 1-mm spacers and consisted of 6% polyacrylamide (19:1 acrylamide:bisacrylamide, by weight; Life Technologies) with a linear denaturing gradient ranging from 0% to 90% denaturant (100% denaturant is 7.0 mol/L urea and 400 mL/L formamide, SigmaUltra; Sigma). PCR products (25-μL samples) were loaded onto the gel and subjected to electrophoresis at 160 V for 4.5 h in a PROTEAN II slab electrophoresis cell (Bio-Rad) submerged in 1× Tris-acetate-EDTA buffer kept at 56 °C. The gels were stained with ethidium bromide and photographed under ultraviolet transillumination. PCR products showing aberrant migration patterns were subjected to direct sequencing.

SEQUENCING

PCR products for sequencing were produced in 100-μL reactions using 2 U of *Taq* polymerase (Qiagen) with 200 ng of DNA, 20 pmol of each primer, and 50 μmol/L dNTPs. Primers for sequencing were identical to primers for DGGE but were without GC clamps. Three microliters of the PCR product was sequenced with the Thermo Sequenase™ Cycle Sequencing Kit (Amersham Life Science), according to the manufacturer's instructions. Sequencing products were run in sequencing gels (6% polyacrylamide and 7 mol/L urea), which were subsequently dried and inspected using PhosphorImaging Technology (Molecular Dynamics).

cDNA ANALYSIS

RNA was isolated from $\sim 2 \times 10^6$ Epstein-Barr virus-transformed lymphocytes using the Purescript™ RNA Isolation Kit (Gentra Systems). Reverse transcription was performed using Superscript II™ (Life Technologies) in a 20-μL reaction with 150 pmol of random hexamer primers. PCR was carried out using the Expand™ High Fidelity PCR System (Boehringer Mannheim) in a 25-μL reaction containing 1.5 mmol/L MgCl₂, 200 μmol/L dNTPs, 20 pmol of each primer, 2 U of *Taq* polymerase, and 2 μL of first-strand cDNA. The amplification was carried out with the following cycling conditions: 1 min at 94 °C; 40 rounds of 94 °C (30 s), 58 °C (30 s) and 72 °C (30 s); followed by a step of 72 °C for 7 min. Reverse transcription-PCR products were run on a 20% premade polyacrylamide gel (Novex) in 1× Tris-borate-EDTA buffer at 200 V for 4 h, stained with ethidium bromide, and photographed under ultraviolet light. Bands of interest were excised, and products were eluted in 100 μL of distilled H₂O overnight and sequenced directly.

Results

In this study we developed a method based on a combination of PCR and DGGE for rapidly detecting mutations and small deletions and insertions in the six exons and the surrounding intronic sequences of the *PTS* gene. DGGE exploits differences in the thermal stability of DNA molecules caused by differences in their primary DNA sequence as a result of mutations. Appropriate PCR-mediated addition of a GC-rich sequence, a "GC clamp" (usually 40 bp in length), to one end of the PCR product modulates the melting profile of the PCR product into the two-domain profile that is considered optimal for detecting single-base changes (20–22).

PRIMER DESIGN

Primers for amplification of individual *PTS* exons were positioned in the adjacent intronic sequences, aiming at

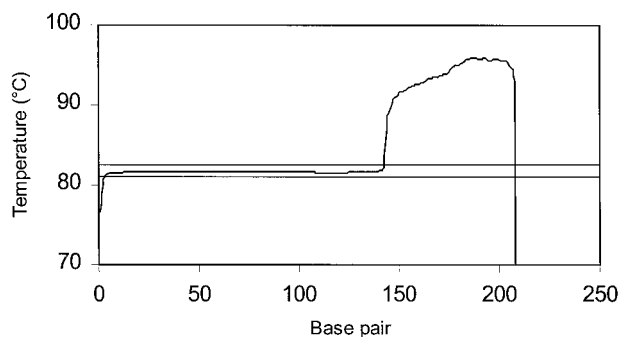


Fig. 1. Melting map of a 202-bp product containing the whole of *PTS* exon 1 and a 56-bp GC clamp at the 3' end.

The horizontal lines indicate the interpolated temperatures at which the calculated log dissociation constant is -5 for the PCR product with a 40-bp clamp (lower line) or a 56-bp clamp (upper line).

the following criteria: (a) all PCR products should have a two-domain melting profile with the sequence of interest contained within the low-melting domain; (b) all PCR products should be of similar size; and (c) all PCR products should withstand prolonged electrophoresis in a denaturing gradient gel. Generation of amplification primers gave rise to two problems: one relating to the very high GC content of exon 1; the other relating to a 15-bp poly(dT) stretch in the splice acceptor site of intron 1.

PTS EXON 1 IS GC RICH AND HIGH MELTING

For analysis of *PTS* exon 1, a standard 40-bp GC clamp was initially incorporated into the 3' end of the PCR product to generate the two-domain melting profile. Despite these theoretical precautions, DGGE analysis of the amplification product failed to produce band focusing and mutation resolution (data not shown). Closer examination of the thermodynamic properties of the amplified sequence by use of the MELT87 algorithm showed that the temperature at which strand dissociation of the GC-clamped amplification product becomes significant was

below the melting temperature of the lower-melting domain. The low temperature of strand dissociation implies direct transition of the amplification product from the fully helical state to the fully single-stranded state, thereby bypassing the partially melted state and preventing resolution of mutations by DGGE. To increase the temperature of strand dissociation above the melting temperature of the lower-melting domain, the length of the GC clamp was increased to 56 bp (Fig. 1). In accordance with the theoretical considerations, DGGE analysis allowed band focusing and clear resolution of the control mutation.

POLY(dT) STRETCH IN INTRON 1

For analysis of *PTS* exon 2, primers were initially positioned ~ 30 bp upstream and downstream of exon 2, respectively. Despite the optimal melting properties of the PCR product generated with these primers, as evaluated by computerized simulation, DGGE analysis revealed at least four distinct bands for all wild-type control samples (Fig. 2A), and resolution of the control mutation was virtually impossible (Fig. 2A, lane 4). We speculated that this unwanted band pattern might relate to a 15-bp poly(dT) stretch that is located immediately upstream of exon 2 as part of the polypyrimidine tract of the splice acceptor site of intron 1. Previous studies have shown that microsatellite repeats and mononucleotide runs are susceptible to insertions and deletions during amplification *in vitro* and *in vivo* because of "slipped strand mispairing" or recombination mechanisms (23–25). The generation of frameshift products and heteroduplexes differing in melting temperature from that of the wild-type sequence would explain the observed multiplicity of bands in a denaturing gradient gel.

Because modifications of different components of the PCR, including the thermostable DNA polymerase, have little effect on the fidelity of microsatellite repeat synthesis

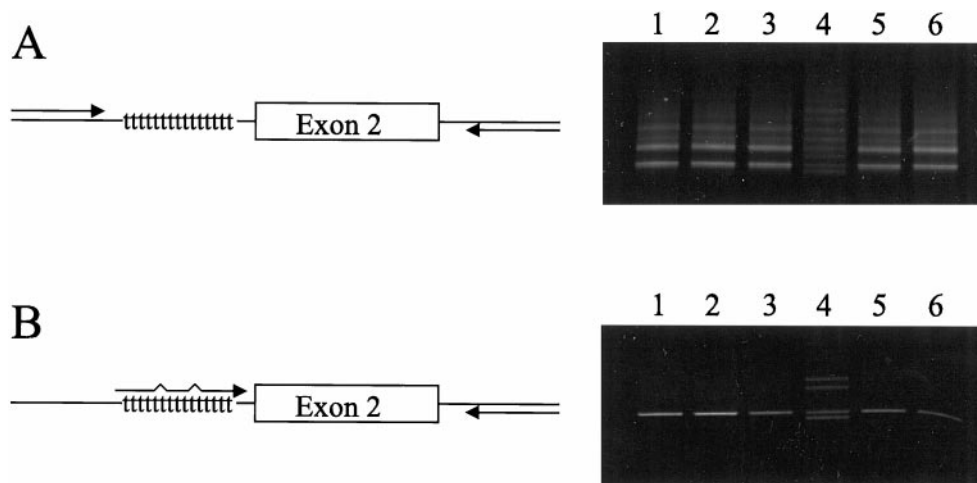


Fig. 2. PCR/DGGE analysis of *PTS* exon 2.

(A), DGGE analysis of PCR products generated with a forward primer located upstream of the poly(dT) stretch. (B), DGGE analysis of PCR products generated with a forward primer containing two mismatches and encompassing the poly(dT) stretch. Lanes 1–3, 5, and 6, wild-type DNA; lane 4, DNA containing mutation N47D.

(26), we sought to modify the upstream primer to decrease the production of frameshift products using standard *Taq* polymerase. The most successful approach is delineated in Fig. 2B. A primer encompassing the entire poly(dT) stretch was designed with two mismatches that cause the substitution of the 5th and 10th thymidines of the stretch with guanosine and cytosine, respectively. The use of this primer together with an unmodified downstream primer allowed successful amplification under standard PCR conditions, generating a product that resolved as a single band in the denaturing gradient gel for all wild-type control samples and as a characteristic four-band pattern for the mutant control sample (Fig. 2B, lane 4).

ONE-STEP ANALYSIS OF *PTS*

To establish one common protocol for PCR amplification of all six exonic regions of *PTS*, the annealing temperature and the concentrations of $MgCl_2$, primers, and dimethyl sulfoxide were varied. At an annealing temperature of 58 °C, all six regions were amplified with sufficiently high yields to allow subsequent DGGE analysis, although these conditions were suboptimal for exon 2 (optimum annealing temperature, 55 °C).

For simultaneous scanning of all six PCR products in a single DGGE run, denaturing gradient range, buffer temperature, and electrophoresis time were varied. Optimal resolution of control mutations was obtained in a 0–90% denaturing gradient gel kept at a constant temperature of 56 °C and run at 160 V for 4.5 h (Fig. 3). The electrophoresis time needed for simultaneous analysis of the six PCR products is the time it takes for all products to reach the positions in the gel where they undergo partial melting and consequently are retarded. Using these standardized

PCR and DGGE conditions, all control mutations were readily resolved.

ANALYSIS OF PATIENTS

Using the method outlined above, we characterized eight mutant alleles in five individuals, including two siblings, of whom four had been clinically diagnosed with PTPS deficiency and one was a putative carrier (Table 1).

In patient A, the mutations V57del and IVS2-7T→A were identified. A survey of the literature suggested that this patient had been studied previously by others (27). V57del is a deletion of the nucleotides 178–180, producing an in-frame deletion of residue 57 (valine). This mutation causes the hexameric structure of the PTPS enzyme to destabilize and aggregate into higher molecular structures (28). IVS2-7T→A causes skipping of exon 3 and the introduction of a premature stop codon (13). The severe phenotype of this patient corresponds well with the dramatic impact of these mutations on the PTPS enzyme.

The sibling patients B and C were found to be homozygous for the IVS1-3C→G mutation. This splice defect previously has been described to cause a complete inactivation of the enzyme (29), which is in concordance with the severe phenotype of these patients.

The IVS1-3C→G mutation was also identified on one of the chromosomes of patient D, who belongs to the mild/atypical group of PTPS-deficient patients with transient HPA. PCR/DGGE analysis did not reveal mutations on the other chromosome. The PTPS activity and urinary neopterin and biopterin concentrations of the patient suggested a defect in the *PTS* gene on both chromosomes. Sequence analysis of all coding regions of the *PTS* gene confirmed the absence of additional mutations.

In patient E, one previously undescribed mutation, V97M (289G→A) (7599G→A; NCBI GenBank accession no. L76259), was detected. No mutations were identified on the other chromosome, which is in concordance with the biochemical diagnosis of heterozygosity based on normal neopterin and biopterin concentrations (Table 1). Obligate heterozygosity previously has been described as a cause of HPA (30). The impact of V97M on the PTPS enzyme function remains to be determined.

The IVS1-3C→G mutation identified in patients B, C, and D previously has been reported to cause the deletion of four codons (nucleotides 84–91) via a cryptic splice site in exon 2 (29). However, electrophoresis of reverse transcription-PCR products generated with primers located in *PTS* exons 1 and 2, respectively, showed a multitude of bands in patients homozygous and heterozygous for this mutation (data not shown). Sequence analysis of individual bands revealed a large number of different splice variants in addition to the previously reported 12-bp deletion. For example, one of the aberrantly spliced transcripts contained an insertion of 4 bp (TGAG) between exons 1 and 2. This corresponds to the highly unusual usage of a dinucleotide GG, at positions –5 and –6 of IVS1, as acceptor splice site instead of the wild-type,

Exon 1 Exon 2 Exon 3 Exon 4 Exon 5 Exon 6
N M N M N M N M N M N M

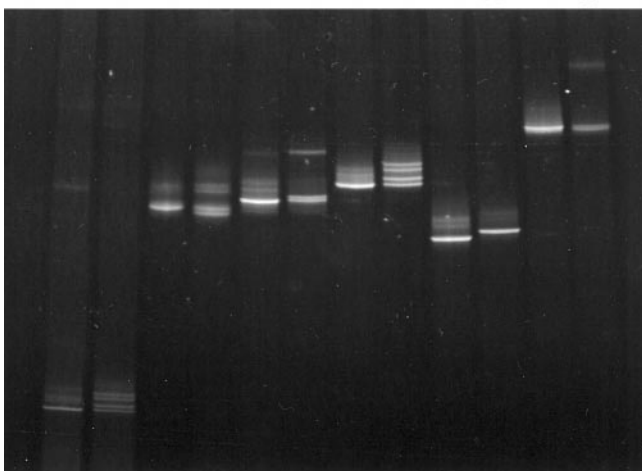


Fig. 3. Simultaneous PCR/DGGE analysis of all six *PTS* exons.

N, wild-type DNA; M, mutant DNA. Mutations are R16C (heterozygous) in exon 1, N47D (heterozygous) in exon 2, V57del/IVS2-7T→A (genetic compound) in exon 3, T67M (heterozygous) in exon 4, P87L (homozygous) in exon 5, and D136V (heterozygous) in exon 6.

unaffected AG dinucleotide, underscoring the importance of a cytosine at position -3 for maintaining the function of the normal splice site.

Discussion

DNA-based diagnostic procedures are particularly useful for diagnosis of diseases that may share phenotypic features with other conditions. Neonatal HPA, which is the initial observation in PTPS deficiency, is also a feature of other metabolic disorders, including phenylketonuria, GTP cyclohydrolase I deficiency, and dihydropteridine reductase deficiency (2). Although the vast majority of cases showing HPA are PAH deficiencies that can be adequately treated by a phenylalanine-restricted diet, rapid and correct diagnosis of cases of BH₄ deficiency is important because particular therapeutic requirements are needed. A genetic test that determines mutations in the *PTS* gene provides a simple routine analysis that directly identifies PTPS deficiency as the cause of HPA. In addition, a DNA-based test provides a sensitive and reliable method for prenatal diagnosis.

The PCR/DGGE-based method presented in here fulfills several criteria that make it suitable for diagnostic applications. First, it has the potential to detect all possible alterations within the regions analyzed, irrespective of site and type of mutation. The high specificity was achieved through careful design of amplification primers, producing GC-clamped PCR products with two-domain melting profiles. Second, the method is designed to allow simultaneous examination of all exons and exon-intron boundaries of *PTS* by applying standardized PCR and DGGE conditions. Thus, the entire procedure may be performed in a single day with minimum hands-on time. All sequence variants detected by DGGE eventually must be identified by sequence analysis to confirm a likely effect on enzyme function and to exclude possible silent variants. We have screened >108 wild-type *PTS* alleles and found only 1 silent variant (T→C at codon 135) at a frequency of 4%, suggesting that the *PTS* gene is not polymorphic and that most sequence variants detected by DGGE-based mutation scanning are likely to be disease causing. To date, 31 mutations associated with PTPS deficiency are registered in the Database of Mutations Causing Tetrahydrobiopterin Deficiencies (31). Sixteen of these mutations were reviewed by Thöny and Blau (32), two were described by Liu et al. (33), two by Scherer-Oppliger et al. (10), and the remaining have been submitted online.

We have used the present method to examine the *PTS* gene in five cases clinically diagnosed as having PTPS deficiency. In all patients with severe PTPS deficiency, two mutations were identified in homoallelic or heteroallelic constellations. In the two remaining cases, only one mutant allele could be characterized. In both cases, the entire coding sequence and splice sites of *PTS* were sequenced to confirm the negative results of the DGGE-based scanning. Case E had relatively low PTPS activity in

erythrocytes but had urinary neopterin and biopterin concentrations within the reference range, suggesting that this patient may be a heterozygote. The other case (D) with only one identifiable mutation, had a more severe, albeit transient course [described in detail by Güttler et al. (6)], and both parents had enzyme activities and urine pterin values within the reference range for heterozygotes (data not shown). Sequence analysis of the parents revealed the father as the carrier of the IVS1-3C→G mutation, whereas no mutation was detected in the mother. The discrepancy between mutation findings and biochemical phenotypes in the patient and her mother could possibly be explained by the presence of a mutation outside the regions analyzed in this study. Mutations located far into the intronic regions may have an influence on the normal splicing mechanism. In addition, the expression of an allele could be affected by a mutation in the promoter region. The latter mechanism has been suggested in a case of severe PTPS deficiency, where analysis of the cDNA indicated homozygosity for the mutation T67M, whereas analysis of the genomic DNA revealed heterozygosity for the same mutation (29). A reduced or absent expression of the allele not carrying the T67M mutation could explain this phenomenon. We also attempted analysis of cDNA to determine whether the apparent heterozygosity in patient D was associated with lowered expression of the other allele. However, this analysis was inconclusive because the multitude of splice variants caused by the IVS1-3C→G mutation made it impossible to determine the degree of expression of the wild-type allele in this patient.

In conclusion, we have developed a fast and inexpensive method for the detection of small mutations/insertions/deletions in exons and exon-intron boundaries of the *PTS* gene. This method should be useful for diagnosis and prenatal diagnosis as well as for large-scale screening studies of PTPS deficiencies.

This work was supported by The Danish Health Insurance Foundation (11/224-98), The Danish Medical Research Counsel (28809-9503008), The Plasmid Foundation, The Novo Foundation, Franz Hoffmann's Memorial Fund, Ernst and Vibeke Husman's Fund, and Direktør Jacob Madsen and hustru Olga Madsen's Fund. We wish to thank Karen Friis Henriksen for technical assistance, Drs. Beat Thöny (University Children's Hospital, Zurich, Switzerland) and Harvey L. Levy (Children's Hospital and Harvard Medical School, Boston, MA) for providing DNA samples with known *PTS* mutations, and Dr. Inge-Merete Nielsen (County Hospital of South West Zealand, Denmark) for referring two patients (patients B and C).

References

1. Kaufman S. Phenylketonuria and its variants [Review]. *Adv Hum Genet* 1983;13:217-97.

2. Scriver CR, Kaufman S, Eisensmith RC, Woo SLC. The hyperphenylalaninemias. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. The metabolic and molecular bases of inherited disease, 7th ed. New York: McGraw-Hill, 1995:1015–75.
3. Nagatsu T, Ichinose H. Regulation of pteridine-requiring enzymes by the cofactor tetrahydrobiopterin [Review]. *Mol Neurobiol* 1999; 19:79–96.
4. Blau N, Dhondt J. International database of tetrahydrobiopterin deficiencies. <http://www.unizh.ch/~blau/biodef1.html> (accessed July 1999).
5. Blau N, Blaskovics M. Hyperphenylalaninemias. In: Blau N, Duran M, Blaskovics M, ed. Physician's guide to the laboratory diagnosis of metabolic diseases, 1st ed. Oxford: Chapman & Hall, 1996: 65–78.
6. Güttler F, Lou H, Lykkelund C, Niederwieser A. Combined tetrahydrobiopterin-phenylalanine loading test in the detection of partially defective biopterin synthesis. *Eur J Pediatr* 1984;142:126–9.
7. Blau N, Kierat L, Matasovic A, Leimbacher W, Heizmann CW, Guardamagna O, Ponzzone A. Antenatal diagnosis of tetrahydrobiopterin deficiency by quantification of pterins in amniotic fluid and enzyme activity in fetal and extrafetal tissue. *Clin Chim Acta* 1994;226:159–69.
8. Blau N, Thöny B, Heizmann CW, Dhondt JL. Tetrahydrobiopterin deficiency: from phenotype to genotype [Review]. *Pteridines* 1993; 4:1–10.
9. Blau N, Thöny B, Spada M, Ponzzone A. Tetrahydrobiopterin and inherited hyperphenylalaninemias [Review]. *Turk J Pediatr* 1996; 38:19–35.
10. Scherer-Oppliger T, Matasovic A, Laufs S, Levy HL, Quackenbush EJ, Blau N, Thöny B. Dominant negative allele (N47D) in a compound heterozygote for a variant of 6-pyruvoyltetrahydropterin synthase deficiency causing transient hyperphenylalaninemia. *Hum Mutat* 1999;13:286–9.
11. Ponzzone A, Blau N, Guardamagna O, Ferrero GB, Dianzani I, Endres W. Progression of 6-pyruvoyl-tetrahydropterin synthase deficiency from a peripheral into a central phenotype. *J Inherit Metab Dis* 1990;13:298–300.
12. Thöny B, Leimbacher W, Bürgisser D, Heizmann CW. Human 6-pyruvoyltetrahydropterin synthase. cDNA cloning and heterologous expression of the recombinant enzyme. *Biochem Biophys Res Commun* 1992;189:1437–43.
13. Kluge C, Brecevic L, Heizmann CW, Blau N, Thöny B. Chromosomal localization, genomic structure and characterization of the human gene and a retropseudogene for 6-pyruvoyltetrahydropterin synthase. *Eur J Biochem* 1996;240:477–87.
14. Thöny B, Heizmann CW, Mattei MG. Chromosomal location of two human genes encoding tetrahydrobiopterin-metabolizing enzymes: 6-pyruvoyl-tetrahydropterin synthase maps to 11q22.3-q23.3, and pterin-4 α -carbinolamine dehydratase maps to 10q22. *Genomics* 1994;19:365–8.
15. Bürgisser DM, Thöny B, Redweik U, Hess D, Heizmann CW, Huber R, Nar H. 6-Pyruvoyl tetrahydropterin synthase, an enzyme with a novel type of active site involving both zinc binding and an intersubunit catalytic triad motif; site-directed mutagenesis of the proposed active center, characterization of the metal binding site and modelling of substrate binding. *J Mol Biol* 1995;253: 358–69.
16. Shintaku H, Niederwieser A, Leimbacher W, Curtius HC. Tetrahydrobiopterin deficiency: assay for 6-pyruvoyl-tetrahydropterin synthase activity in erythrocytes, and detection of patients and heterozygous carriers. *Eur J Pediatr* 1988;147:15–9.
17. Grimberg J, Nawoschik S, Belluscio L, McKee R, Turck A, Eisenberg A. A simple and efficient non-organic procedure for the isolation of genomic DNA from blood. *Nucleic Acids Res* 1989; 17:8390.
18. Rychlik W, Rhoads RE. A computer program for choosing optimal oligonucleotides for filter hybridization, sequencing and in vitro amplification of DNA. *Nucleic Acids Res* 1989;17:8543–51.
19. Lerman LS, Silverstein K. Computational simulation of DNA melting and its application to denaturing gradient gel electrophoresis. *Methods Enzymol* 1987;155:482–501.
20. Abrams ES, Stanton VP. Use of denaturing gradient gel electrophoresis to study conformational transitions in nucleic acids. *Methods Enzymol* 1992;212:71–104.
21. Sheffield VC, Cox DR, Lerman LS, Myers RM. Attachment of a 40-base-pair G+C-rich sequence (GC-clamp) to genomic DNA fragments by the polymerase chain reaction results in improved detection of single-base changes. *Proc Natl Acad Sci U S A* 1989;86:232–6.
22. Myers RM, Maniatis T, Lerman LS. Detection and localization of single base changes by denaturing gradient gel electrophoresis. *Methods Enzymol* 1987;155:501–27.
23. Odelberg SJ, Weiss RB, Hata A, White R. Template-switching during DNA synthesis by *Thermus aquaticus* DNA polymerase I. *Nucleic Acids Res* 1995;23:2049–57.
24. Levinson G, Gutman GA. Slipped-strand mispairing: a major mechanism for DNA sequence evolution [Review]. *Mol Biol Evol* 1987;4:203–21.
25. Meyerhans A, Vartanian JP, Wain-Hobson S. DNA recombination during PCR. *Nucleic Acids Res* 1990;18:1687–91.
26. Hite JM, Eckert KA, Cheng KC. Factors affecting fidelity of DNA synthesis during PCR amplification of d(C-A)_n · d(G-T)_n microsatellite repeats. *Nucleic Acids Res* 1996;24:2429–34.
27. Oppliger T, Thöny B, Leimbacher W, Schreiberreiter S, Brandt NJ, Heizmann CW, Blau N. Mutation analysis in patients with 6-pyruvoyl-tetrahydropterin synthase deficiency. *Pteridines* 1995; 6:141–3.
28. Oppliger T, Thöny B, Nar H, Bürgisser D, Huber R, Heizmann CW, Blau N. Structural and functional consequences of mutations in 6-pyruvoyltetrahydropterin synthase causing hyperphenylalaninemia in humans. *J Biol Chem* 1995;270:29498–506.
29. Oppliger T, Thöny B, Kluge C, Matasovic A, Heizmann CW, Ponzzone A, et al. Identification of mutations causing 6-pyruvoyltetrahydropterin synthase deficiency in four Italian families. *Hum Mutat* 1997;10:25–35.
30. Niederwieser A, Shintaku H, Leimbacher W, Curtius HC, Hyànek J, Zeman J, Endres W. "Peripheral" tetrahydrobiopterin deficiency with hyperphenylalaninaemia due to incomplete 6-pyruvoyl tetrahydropterin synthase deficiency or heterozygosity. *Eur J Pediatr* 1987;146:228–32.
31. Blau N, Thöny B, Dianzani I. Database of mutations causing tetrahydrobiopterin deficiencies. <http://www.unizh.ch/~blau/biomdb1.html> (accessed July 1999).
32. Thöny B, Blau N. Mutations in the GTP cyclohydrolase I and 6-pyruvoyl-tetrahydropterin synthase genes [Review]. *Hum Mutat* 1997;10:11–20.
33. Liu TT, Hsiao KJ, Lu SJ, Wu SJ, Wu KF, Chiang SH, et al. Mutation analysis of the 6-pyruvoyl-tetrahydropterin synthase gene in Chinese hyperphenylalaninemia caused by tetrahydrobiopterin synthesis deficiency. *Hum Mutat* 1998;11:76–83.