A mutation causing DHPR deficiency results in a frameshift and a secondary splicing defect

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Abstract
In our analysis of mutations causing DHPR deficiency we identified a patient in whom there was an aberrant transcription pattern detected by PCR of DHPR cDNA. However, unlike the pattern observed as a result of most splicing mutations, there is some full length transcript. The mutation was located and is a single nucleotide deletion at position 570/571 of the DHPR cDNA sequence and results in a frameshift and premature termination after the addition of six amino acids. The mutation is present in a homozygous state in the patient and in a heterozygous state in both parents. The exon which is deleted at high frequency in the patient is the putative exon 4, which is remote from the mutation, and confirms our observation that exon 4 skipping is a relatively common event.


Dihydropteridine reductase deficiency is a rare inherited disorder characterised by hyperphenylalaninaemia and progressive neurological dysfunction not amenable to treatment by limitation of serum phenylalanine.1 The disease is the result of a deficiency of the electron donating cofactor, tetrahydrobiopterin, required for the aromatic amino acid hydroxylases.2 Thus, in addition to a reduction in the hydroxylation of phenylalanine, and the concomitant increase in serum levels, there is also a reduction in the hydroxylation of tryptophan and tyrosine, resulting in decreased levels of serotonin and dopamine.3 This reduction in neurotransmitter levels leads to a Parkinsonian type syndrome, often with frequent seizures as the cause of death in untreated cases.

Enzyme studies pinpointed dihydropteridine reductase (DHPR, EC 1.6.99.7) as being defective in patients with the deficiency. Thus, in an analysis of one family with the disorder, cultured fibroblasts of the parents exhibited heterozygote levels of activity and those of the affected child had no activity.4 Further investigations showed that there are two classes of DHPR deficient patients: those with cross reacting material (CRM) in fibroblasts (of varying levels) and those without.5 The cloning of the DHPR cDNA6 enabled mutations within the coding regions of DHPR deficient patients to be identified, and shortly thereafter the first such mutation was reported.7 Since this time we have reported nine further mutations7–10 with another reported by Matsubara et al.11 The mutations are spread throughout the coding region, and their heterogeneous nature was confirmed by the analysis of several mutant proteins expressed in E coli.10 A review of the mutations is found in Smooker and Cotton.12

During the analysis of fibroblast RNA several apparent splicing errors were identified. Two of these were examined further and found to be the result of donor site mutations at the intron/exon boundaries of the DHPR gene12 (Smooker, Gough, and Cotton, manuscript in preparation). In this report we describe a third family which carries a mutation causing a similar molecular phenotype, but we show that the effect of the mutation on splicing is not the primary effect.

Clinical summary
The proband was the first child of Pakistani parents, who were first cousins. She was born at 42 weeks' gestation after an uncomplicated pregnancy, with a birth weight of 3300 g. There were no apparent problems in the immediate perinatal period and she was discharged on day 3. A blood spot phenylalanine analysis performed on day 3, while she was breast fed, was raised to 400 µmol/l (normal <240), with the repeat level on day 11 being 440, and being 720 by day 16. She was admitted on day 20 for a biochemical evaluation of hyperphenylalaninaemia. An oral tetrahydrobiopterin (BH₄) load (20 mg/kg) showed a dramatic fall in the plasma phenylalanine level (preload level 1059 µmol/l, three hour postload level 914, 6.5 hour postload 586, 11 hour 401, 24 hour 298). Urinary pterin analysis before the load showed only a trace of BH₄ (normally 80% of total bipterin is BH₄).3 CSF neurotransmitter levels were low: CSF 5-hydroxyindolacetic acid was 5·43 ng/ml (normal 81 ± 29), while homovanillic acid was 19·3 ng/ml (normal 71 ± 15). Assay of dried bloodspot DHPR showed no measurable activity on two samples collected several days apart. These studies established the diagnosis of DHPR deficiency. Treatment consisting of dietary restriction of phenylalanine and supplementation with L-dopa (9 mg/kg/day), carbidopa (1 mg/kg/day), 5-hydroxytryptophan (7·5 mg/kg/day), and folic acid (12·5 mg bid) was started. Over the next 6 months growth and development were normal. The serum phenylalanine level ranged from 240 to 530 µmol/l (recommended therapeutic range 250 to 600).

At 6 months of age BH₄ (12 mg/kg/day) was added to the drug regimen, allowing the dietary phenylalanine intake to be liberalised without...
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compromising plasma phenylalanine control. Height and weight velocity were maintained, while head growth decelerated, resulting in microcephaly. When last reviewed at 3 years 8 months, her weight was 19.6 kg (>97th centile), height was 99.5 cm (50th to 75th centile), and head circumference was 47 cm (2nd centile). Her phenylalanine tolerance was estimated at 42 mg/kg/day while on BH4, replacement therapy, her most recent plasma phenylalanine level being 387 μmol/l.

Materials and methods

ISOLATION OF NUCLEIC ACIDS

Cultured skin fibroblasts, obtained from a forearm skin biopsy, were grown to confluence in MEM medium supplemented with 10% fetal calf serum. For the purification of RNA or DNA, cells were recultured in basal Eagle’s medium, and approximately 2 × 10⁸ cells were harvested, snap frozen in liquid nitrogen, and stored at −70 °C until use. The isolation of RNA, conversion to first strand cDNA, and PCR amplification were performed as previously described.²⁴ RNA was also isolated from frozen lymphocytes (from the parents) and from a chorionic villus sample (CVS) from a subsequent pregnancy in the family, using identical extraction procedures. From 0.1 to 5 μg of total RNA was used to produce cDNA (the amount depending on the source, with very little RNA available from lymphocytes). Approximately 10 to 25% of the 20 μl cDNA reaction was used for PCR.

AMPLIFICATION AND ANALYSIS OF GENOMIC DNA

Genomic DNA was isolated from lymphocytes and fibroblast cultures by the method of Miller et al.²⁴ PCR amplification across intron 4 of the DHPR gene used primers 480 and 610 (5’GGCAGCGGTGCTGTTCA3’ and F were used. The products were electrophoresed in agarose gels and, if required for DNA sequencing, eluted from the gels. DNA sequencing used a Sequenase kit (United States Biochemicals) and was performed according to the method of Bachmann et al.¹³

Results

CHARACTERISATION OF DHPR TRANSCRIPTS BY PCR ANALYSIS

RNA was isolated from cultured fibroblasts from the patient, cultured fibroblasts from a control (non-DHPR deficient), lymphocytes from the two parents, and from a CVS from the latest pregnancy of the family. RFLP analysis showed that the family was semi-informative, and the CVS was shown to carry at least one normal allele (Smooker, unpublished data). The DHPR coding region was amplified in two overlapping halves, using the oligonucleotide pairs GD and 132 and 131 and F. Results are shown in fig 2. In the 5’ region of the DHPR cDNA there is a full length product in all cases; however at the 3’ end there is aberrant transcription in the patient (lane 5), where the major product is a smaller band of approximately 320 bp compared to the normal size of 430 bp. The normal sized cDNA was nevertheless present. Conversely there is also some of the smaller product in the normal sample (lane 6), as we have previously observed (Smooker, Gough, and Cotton, manuscript in preparation). We have shown that the small product lacks exon 4.

SEQUENCE ANALYSIS OF THE AMPLIFIED TRANSCRIPTS

The nucleotide sequence of the 320 bp amplified cDNA product from the patient was determined (fig 3A), and found to have a 110 bp deletion when compared to the wild type cDNA clone previously isolated in our laboratory.⁴ The exact site of the boundaries could not be determined (owing to their occurrence within a run of cytosine residues); however they are between 460/461 and 570/571, inclusive. This result is similar to the pattern we have previously observed in other patients with splicing errors,¹² except that an additional nucleotide is missing. In that study, we found a deletion of 109 nucleotides in the same region, and showed that this deletion was the result of the skipping

![Diagram of DHPR gene and oligonucleotides](image)
As we showed using PCR, however, the exon skipping is not complete, and there is some full length transcript present. The nucleotide sequence of this product amplified from patient cDNA was obtained, and is shown in fig 3B, compared to the wild type sequence. The wild type sequence was derived from RNA isolated from the CVS, and is identical in this region to that of our reference cDNA clone. This observation confirms that the fetus carries at least one normal allele, as expected from the result obtained by RFLP analysis (Smooker, unpublished data). In the sequence from patient cDNA, there is a single nucleotide deletion, one of the two G nucleotides at position 570/571 in the cDNA sequence. The deletion of this nucleotide will cause a frameshift and the addition of six amino acids before termination. Hence, although there is some full length transcript in the patient, it will not encode normal DHPR protein. This mutation (delG570/571) was tentatively assigned as causative in this family. The position of the mutation is at the boundary of intron 4/exon 5, which has a triplet of G nucleotides spanning the boundary (see below).

CHARACTERISATION OF DHPR GENOMIC DNA
In order to characterise this mutation further, genomic DNA was studied near the boundary of intron 4 and exon 5. This region was amplified using exon primers 480 and 610 to amplify across the entire intron 4. As expected, no large deletions were found in the genomic DNA (data not shown). The nucleotide sequences of the amplified products were obtained and are shown in fig 4. These results show that the patient is homozygous for the deleted nucleotide described above, and that both parents are carriers for this mutation. This confirms that the delG570/571 mutation is present throughout this family, and is highly likely to be the cause of DHPR deficiency. As it is not possible to determine which G is deleted, the assignment of the mutation as an exon mutation is arbitrary. A summary of the findings described here is found in fig 1.

Discussion
In this report we describe a new mutation resulting in the rare metabolic disorder DHPR deficiency. This mutation is a single nucleotide deletion, and results in a frameshift followed by premature termination, and a secondary splicing defect in which the putative exon 4 of the DHPR gene is deleted at a high frequency. Thus, although cDNA from the patient contains some full length transcript, as determined by PCR (fig 2), the major band represents transcripts which have exon 4 plus one of the first nucleotides of exon 5 deleted. The observation that the parents (who are heterozygous for the mutation) have very little of the deleted transcript detectable by PCR, compared to that of the full length species, suggests that the expression of deleted transcripts is low. The nucleotide sequence of genomic DNA (fig 4) shows that the patient has
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Figure 4  Sequence of genomic DNA amplified with 480 and 610. The acceptor site is shown (non-coding strand, hence the G deletion appears as C). (1) Lambda clone, (2) father, (3) mother, (4) proband. The heterozygous nature of the mutation in both parents is visible. The number refers to the cDNA clone, as in fig 3.

A single base deletion compared to the wild type sequence obtained from a lambda clone covering part of the DHPR gene. The deletion is either at the last nucleotide of intron 4 or one of the first two nucleotides of exon 5 (all of which are Gs). However, the effect is the same regardless of which nucleotide is deleted, and is manifested as a frameshift. We have therefore termed the mutation as an exon mutation, deleting the first or second nucleotide of exon 5 (that is, delG570/571). It is probable that any 181 amino acid DHPR protein which is translated from this allele will be readily degraded, as previously found in the proband in whom a premature termination was found. Additionally, as there is some exon skipping, the amount of full length mRNA available for translation will be considerably reduced in the patient.

The deletion of a single nucleotide cannot directly explain the loss of exon 4 in the majority of transcripts, as the exon which is deleted is not the one in which the mutation is located. It is probable that the single base deletion in the primary transcripts perturbs the general fidelity of splicing and, as we have observed, exon 4 is very prone to skipping, even in wild type transcripts (Smooker, Gough, and Cotton, manuscript in preparation). This skipping is probably because of the relatively low score assigned to the donor site of this intron, as calculated according to Senepathy et al. It should be noted here that this phenomenon is not general among missense mutations in the DHPR gene, as most do not radically alter the pattern of transcripts detected by PCR. We have, however, detected several patients with obvious splicing defects for which mutations are yet to be found (Smooker, unpublished observations).

In summary, a new mutation of the DHPR gene has been identified, which has the dual effect of causing a frameshift with resultant premature termination, and a splicing error as a secondary effect. It is predicted that no active enzyme will be present in this patient, resulting in hyperphenylalaninaemia. Although microcephaly has developed, developmental progress to date has been satisfactory, and shows the importance of early diagnosis and treatment of this disease.

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