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Insertion of an Extra Codon for Threonine Is a Cause of Dihydropteridine Reductase Deficiency

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Summary

The mutation in a patient with dihydropteridine reductase deficiency has been located and characterized. Polymerase chain reaction (PCR) was used to amplify the coding sequence of human dihydropteridine reductase from the messenger RNA of skin fibroblasts. Chemical cleavage of mismatches indicated a mismatched thymine and cytosine at approximately 117 and 147 bases, respectively, from the end of the probe. Cloning and sequencing of the mutant PCR products revealed the insertion of the triplet ACT (threonine), after alanine 122 (base 390). Amplification of a small region around this mutation by using genomic DNA as the PCR target indicates that the mutation is completely within an exon. Unequal crossing-over at the second base in the preceding alanine codon and duplication of the bases CTA may be the mechanism of mutagenesis. The cleavage site 147 bases from the end of the probe corresponded to the conversion of guanine to adenine at base 420 (CTG to CTA) and does not alter the code for leucine. This change, which was also seen in another dihydropteridine reductase–deficient child and in a control subject probably represents a common neutral polymorphism.

Introduction

Dihydropteridine reductase (NADH: quinonoid 6.7-dihydropteridine oxidoreductase; E.C.1.6.99.7) (DHPR) catalyzes the recycling of tetrahydrobiopterin (Craine et al. 1972), the cofactor required for hydroxylation of phenylalanine, tyrosine, and tryptophan (Kaufman 1958; Levitt et al. 1965; Hosoda and Glick 1966). In man, deficiency of this enzyme is inherited as an autosomal recessive trait and causes a variant form of phenylketonuria (PKU) which is not alleviated by a low-phenylalanine diet (Scriver et al. 1989). The disorder is characterized by a progressive neurological degeneration resulting from failure of noradrenalin, dopamine and serotonin synthesis, and secondary inhibition of folate metabolism (Smith et al. 1986).

Despite its importance, little is known of the structure and organization of this protein. The native enzyme exists as a dimer of two identical subunits, each containing four thiol groups which lie in a hydrophobic environment. Although studies with sulphydryl reagents have failed to distinguish a particular thiol as necessary for enzyme activity, protection of these groups by NADH suggests that one of them may play a role in binding of this nucleotide to the enzyme (Armarego et al. 1984). Knowledge of the cDNA and deduced protein sequences have shown that the human and rat enzymes differ in only 10 amino acids, all of which are conservative substitutions (Dahl et al. 1987; Lockyer et al. 1987; Shahbaz et al. 1987). Comparison with human dihydrofolate reductase (hDHFR), which also has pterin- and nicotinamide-derived cofactors as substrates, has revealed DHPR sequences with similarities to DHFR regions known to comprise the binding sites for methotrexate and the nicotinamide cofactor (Lockyer et al. 1987). In particular, Trp^{24} in DHFR, which has been implicated in the binding of nicotinamide (Lai et al. 1982), is found within a small region of high homology centered around Gly^{20} in hDHFR and around Cys^{104} in hDHPR (Dahl et al. 1987), suggesting that this region may have a similar function in both enzymes. Nothing is known of the amino acids involved in binding of the pterin substrate to DHPR.

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Since the mutations in DHPR deficiency appear to be heterogeneous (Firgaira et al. 1981*a;* Dahl et al. 1987), study of the mutant proteins might allow us to gain further information about the active-site amino acids and their function. However, fibroblasts, erythrocytes, and lymphocytes, which are the only tissues readily available from affected children, contain insufficient enzyme for such studies. An approach to this problem is to characterize the mutations, at the DNA level, in patients whose DHPR is inactive but of normal size, charge, and immunoreactivity and where there is a high probability that the mutation affects the active site and then to construct in vitro mutants for expression of large amounts of these mutant enzymes in a bacterial host. In the present report we describe the first characterization of a human DHPR mutation and the genetic analysis of this mutation in other family members.

Experimental Procedures

Patients

Patient KA, of Lebanese origin, was the first child of consanguineous parents. DHPR deficiency was diagnosed by enzyme assay (Firgaira et al. 1980), and electrophoretic and immunological studies revealed a mutant protein of normal size, charge, and immunoreactivity (Firgaira et al. 1981*a,* 1981*b*). The mother (MA), father (FA), and an unaffected brother (BA) who is a carrier for DHPR deficiency were also studied. Patients S116 and KD4 provided unaffected control material.

Poly A$^+$ RNA Isolation

Skin fibroblasts from the DHPR-deficient patient KA and from the control S116 were cultured to approximately 4×10^7 cells/pellet, treated with trypsin, and then washed three times with PBS. The cells were resuspended in 10 ml 10 mM Tris-HCl pH 7.5 containing 1 mM EDTA, 0.1 M NaCl, 0.5% SDS, and 1 mg proteinase K/ml, and were homogenized by passage three times through a 19-gauge needle, and the DNA was sheared by passage through a 25-gauge needle until the viscosity fell. The preparation was then incubated overnight at 37°C with constant agitation. One hundred milligrams oligo (dT) cellulose equilibrated in incubation buffer (described above) was added and mixed gently for 4 h. The cellulose was centrifuged, and the pellet was washed five times with incubation buffer until OD^{260} was <0.05. The oligo (dT) cellulose/ polyA$^+$ RNA was poured into a small column and further washed with incubation buffer until the OD^{260} of the effluent was 0.00. Poly A$^+$ RNA was eluted with 5 × 500 μl 10 mM Tris-HCl pH 7.5 containing 1 mM EDTA and 0.5% SDS. Fractions where the OD^{260}:OD^{280} ratio was >1.8:1.0 were pooled and then were phenol, phenol/chloroform, and chloroform extracted before precipitation of poly A$^+$ RNA with sodium acetate and ethanol. The pellet was dried under vacuum and then was resuspended in H_2O at 0.5 μg/μl. DHPR poly A$^+$ RNA (1 μg/μl) isolated from the liver of patient KD4 provided additional control material.

cDNA Production

One microgram poly A$^+$ RNA was heated to 65°C for 5 min and then cooled rapidly on ice to prevent formation of secondary structure. This was then used for first-strand cDNA synthesis by using a cDNA synthesis kit (Amersham International plc). Residual RNA was hydrolyzed by addition of 1 μl 0.5 M EDTA and 12.5 μl 150 mM NaOH to 20 μl of cDNA and then was heated to 65°C for 1 h. The mixture was neutralized by addition of 12.5 μl 1 M Tris pH 8 and 12.5 μl 200 mM HCl. After addition of 1 μl of 1 mg tRNA/ml as a carrier, the cDNA was precipitated by addition of sodium acetate and ethanol and was washed with 70% ethanol, dried, and resuspended in 15 μl H_2O.

Polymerase Chain Reaction (PCR)

Two pairs of 20mer oligonucleotide primers, A/D and I/F (fig. 1) were synthesized using the phosphoramidite method to allow overlapping amplification of the entire DHPR coding sequence. An additional pair of oligonucleotides (C/H) were synthesized to allow amplification between bases 334 and 417. Each contained equal proportions of AT/CG bases. Their sequence and position within the DHPR coding sequence are shown in figure 1. PCR was performed using the Cetus Perkin–Elmer protocol with final reaction conditions of 50 mM KCl, 10 mM Tris-HCl pH 8.3, 0.01% gelatin, 200 μM each dNTP, 1 μM each primer, 2.0–2.5 units *Taq* polymerase, and 2.5–5.0 ng total cDNA (1 μl)/reaction. For amplification between oligonucleotides A and D a final $MgCl_2$ concentration of 2.5 mM was used, while for both I to F and C to H the optimum was 1.25 mM $MgCl_2$. Thirty-five cycles of denaturation (2 min at 95°C), annealing (2 min at 65°C), and extension (3 min at 72°C) were performed.

Chemical Cleavage of Mismatches (CCM)

To screen for mutation sites within the amplified PCR products, the CCM method described by Cotton et al.

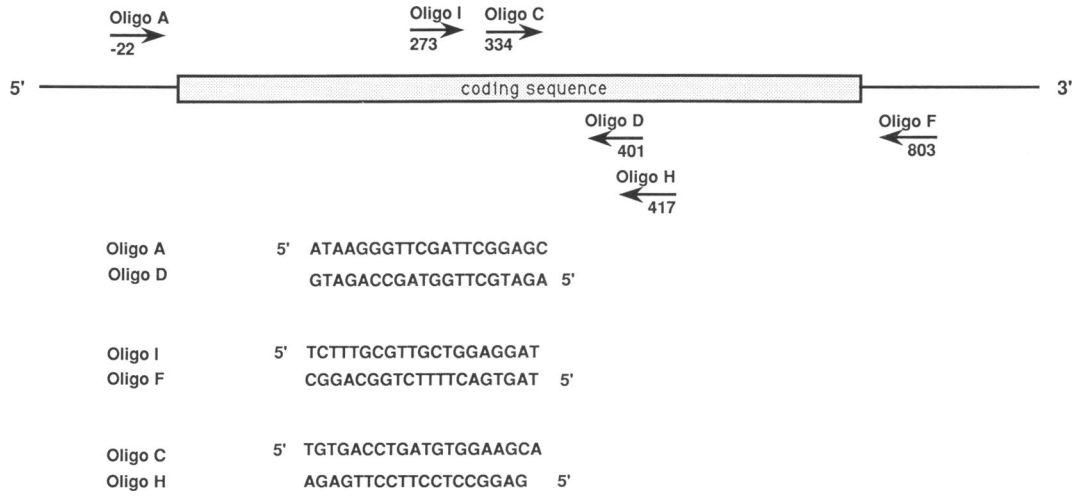

Figure 1 Sequence and position of PCR oligonucleotide primers in relation to hDHPR coding sequence. The position of the 5′ base of the oligonucleotide is indicated.

(1988) and Dahl et al. (1989) was used. In this technique, mutant DNA is allowed to form a duplex with a radiolabeled probe of control DNA. Where mutations are present, the mutant strand does not exactly match the control and fails to anneal correctly. The resulting mismatch is chemically more reactive than the surrounding DNA, allowing chemical cleavage at this site and detection of different-sized radioactive fragments. Because cytosine and thymine react specifically with hydroxylamine and osmium tetroxide (OsO_4), respectively, the nature of the reactivity also gives information about the base change. Since only mismatched cytosines and thymines show marked reactivity, probes in both senses must be used to ensure detection of the complimentary guanine and adenine mismatches.

^{32}P Probe Preparation

Probes for each of the PCR products were prepared by PCR amplification between oligonucleotides A and D and I and F by using cDNA from KD4, an unaffected control, in the presence of 20 pmol ^{32}P dCTP (60 μCi). To allow efficient incorporation of label, the final concentration of nonradioactive dCTP in the reaction was reduced from 200 μM to 6 μM. To prevent misincorporation of alternate dNTPs as [dCTP] fell, only 25 PCR cycles were performed. Probe was then isolated from a 4% nondenaturing acrylamide gel and resuspended in 1 × TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.4) at 1,000 cpm/μl.

Duplex Formation

By means of siliconized eppendorf tubes, homoduplexes (^{32}P-KD4/KD4) and heteroduplexes (^{32}P-KD4/S116 and ^{32}P-KD4/KA) were prepared with 28,000 cpm ^{32}P control DNA (approximately 10.5 ng) and a 10-fold excess of the unlabeled test DNA (105 ng) in 128 μl of H_2O. One hundred twenty-eight microliters of 2 × annealing buffer (12 mM Tris-HCl, 1.2 M NaCl, 14 mM $MgCl_2$, pH 7.7) was added, and the tubes were capped with punctured nonsiliconized caps and boiled for 5 min before being recapped with the siliconized caps and being transferred to 42°C. After 1 h incubation the tubes were removed to ice for 2 min, and the DNA duplex was precipitated in sodium acetate/ethanol and was washed with 70% ethanol, dried, and resuspended at 1,000 cpm/μl in H_2O. Duplexes were frozen at this stage for a few days if required.

Hydroxylamine Reactions

Two reactions were carried out for each duplex. To 6 μl duplex, 20 μl hydroxylamine solution (1.39 g hydroxylamine in 1.6 ml distilled water, adjusted to pH 6.0 by addition of approximately 1 ml diethylamine) was added, and the mixtures incubated for 10 and 60 min, respectively, at 37°C. After incubation, 200 μl "stop solution" (0.3 M sodium acetate pH 5.2, 0.1 mM EDTA, 25 μg tRNA/ml) and 750 μl ethanol were added, and the DNA precipitated at −70°C for 30 min. After

centrifugation the DNA was resuspended in 200 µl 0.3 M sodium acetate pH 5.2, and was mixed, and 500 µl of ethanol was added, and the DNA was reprecipitated, washed with 70% ethanol, and dried.

OsO_4 Reactions

Again two reactions were performed per duplex. To 6 µl duplex, 2.5 µl 10 × OsO_4 buffer (10 mM Tris-HCl pH 7.7, 1 mM EDTA, 1.5% pyridine) and 15 µl of a 1/5 dilution of OsO_4 (0.5 g/12.5 ml distilled water) were added, and the tubes mixed and incubated at 37°C for 1 and 5 min. After incubation the reactions were stopped by addition of 200 µl stop solution (see above), and the DNA precipitated and was dried as for the hydroxylamine reactions.

Piperidine Cleavage of Duplexes

Fifty microliters of 10% piperidine solution was added to each of the dried pellets and vortexed for 10 s, and then the tubes were incubated at 90°C for 30 min and cooled on ice for 2 min. Fifty microliters of 0.6 M sodium acetate pH 5.2 and 300 µl ethanol were added, and the DNA precipitated at −70°C for 30 min and was centrifuged, washed with 70% ethanol, dried, and resuspended at 1,000 cpm/2.5 µl formamide dye (10 mg bromophenol blue, 10 mg xylene cyanol FF, and 0.2 ml of 0.5 mM EDTA dissolved in 10 ml deionized formamide). The samples were boiled for 5 min and cooled on ice, and then 2.5 µl of the sample was subjected to electrophoresis on a 0.4-mm-thick 8% acrylamide/urea denaturing gel run at 35 W until the bromophenol blue marker had run 30 cm.

M13 Cloning and Sequencing

I-F PCR products were cut with *Sma*I to give two fragments. The fragments were treated with T4 polynucleotide kinase to ensure that the 5′-terminae were phosphorylated. The A-D PCR products were not cut with a restriction enzyme but were phosphorylated. Each of these fragments was then ligated into M13mp10 which had been cut with *Sma*I and treated with phosphatase. Sequencing was carried out using the Sequenase protocol.

Results and Discussion

PCR amplification of A-D gave a single well-defined band of 423 bp which cut with the restriction enzyme *Taq*I to give the 271-, 118-, and 34-bp fragments expected of the authentic DHPR sequence. By means of an internally labeled probe of A-D from KD4, CCM in the patient KA gave a background ladder of cytosines and thymines but no specific cleavage products when compared with the control S116 (not shown). Since the CCM method has been shown to detect all classes of single base mutations (Cotton et al. 1988) and small deletions and insertions (Cotton and Campbell 1989), the absence of mismatches indicates the absence of any mutations or polymorphisms in this most 5′ portion of the mutant cDNA.

Amplification of I-F gave a band of 530 bp which gave the expected fragments of 436 and 94 bp when digested with *Taq*I. In addition, a number of nonspecific PCR products whose formation could not be

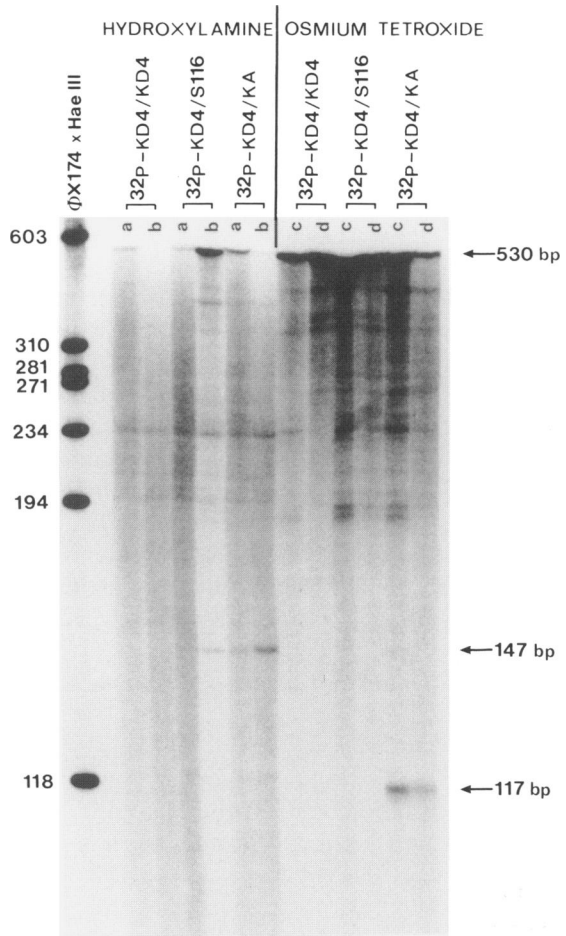

Figure 2 Autoradiogram of CCM when I-F PCR products are used with homoduplex control (32P-KD4/KD4), heteroduplex control (32P-KD4/S116), and mutant heteroduplex (32P-KD4/KA). 1000 cpm were loaded per well of the 8% denaturing gel, which was electrophoresed at 35 W until the bromophenol blue dye had run 30 cm. The reaction times for hydroxylamine were (a) 10 min and (b) 60 min, and those for OsO_4 were (c) 1 min and (d) 5 min.

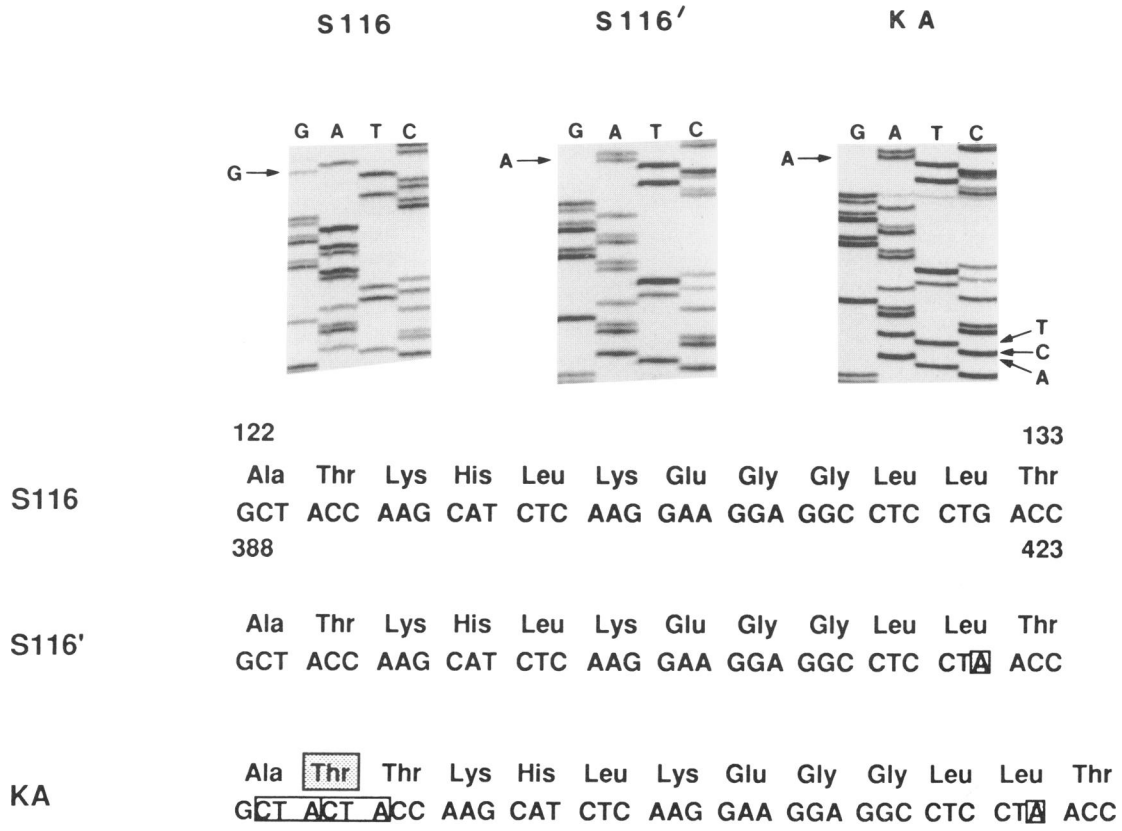

Figure 3 Sequence analysis from base 338 (alanine 122) to base 423 (threonine 133) in the control S116, in a variant clone of the control (S116′) containing the G-A conversion, and in the patient KA with the G-A conversion and the ACT insertion resulting in an extra threonine. The sequence CTA, which appears to have been duplicated, is marked.

prevented by alterations of either PCR annealing temperature or $MgCl_2$ concentration were also present. However, these did not appear to be related to DHPR and did not interfere with the CCM. When internally labeled I-F from KD4 was used as the probe, heteroduplexes with both KA and S116 showed reaction with hydroxylamine, indicating a mismatched cytosine 147 bases from the end of one of the probe strands. The reaction was stronger in KA than in S116 (fig. 2). In addition, KA (but not S116) also reacted with OsO_4, indicating a possible mismatched thymine in the probe strand 117 bases from one end of the probe (fig. 2). Since both probe strands were labeled in this experiment, it was not possible to determine the exact location of these mismatches. Repeating the CCM experiment with a single end-labeled probe would have determined at precisely which end of the I-F fragment these alterations were to be found. However, at this stage *Sma*I digests of the I-F PCR products of KA and S116 had each given two 265-bp fragments that had been cloned into M13; therefore, dideoxy sequencing was used to define the nature of these mismatches.

In KA and S116 the reactivity with hydroxylamine corresponded to a change of a G to an A at base 420 (fig. 3). Although this does not directly involve a C or T, the probe had both sense and antisense strands labeled; thus a C in the antisense strand of the probe incorrectly paired with the A in the sense strand of both KA and S116 to give the chemical reactivity (fig. 4). This change, which was also seen in another DHPR-deficient family (not shown), alters the codon for leucine 132 from CTG to CTA (an alternate codon for leucine) and probably represents a common neutral polymorphism. The lesser intensity of the CCM product from S116 compared with KA suggests that S116 is heterozygous for this polymorphism while KA, who has consanguineous parents, is homozygous. This suggestion is supported by the detection of this base change in all five

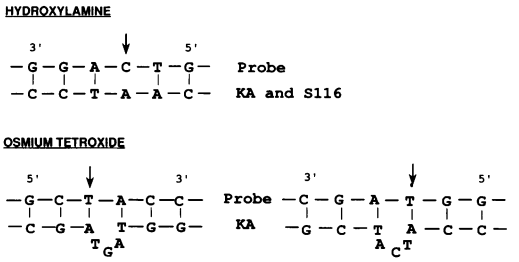

Figure 4 Sequence of heteroduplexes formed between the normal and mutant DNA in the regions surrounding the CTG-to-CTA conversion and the ACT insertion. The cytosine reacting with hydroxylamine in S116 and KA and the thymines in both senses of the probe that react with OsO_4 with KA are indicated by arrows (↓).

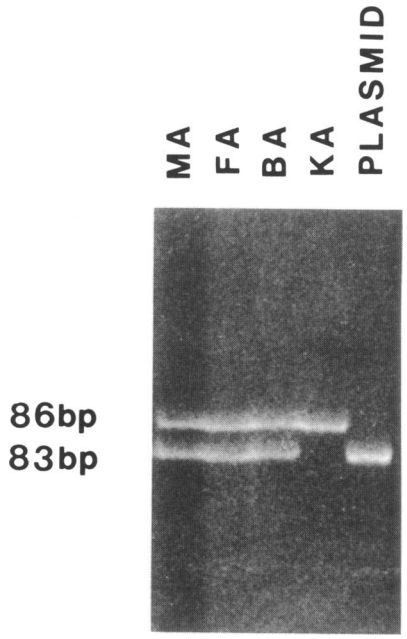

Figure 5 Results on 20% nondenaturing acrylamide gel stained with ethidium bromide. Genomic DNA from the patient KA, the mother MA, the father FA, and the brother BA was used as the target for PCR amplification between the oligonucleotides C and H. Plasmid containing the DHPR cDNA clone was used as the control. KA has only the mutant (86-bp) product, while the mother, father, and brother all have both the mutant and normal (83-bp) sequences.

of the KA clones sequenced but in only one of the two S116 clones.

The band seen with OsO_4 in KA corresponds to insertion of an extra ACT codon between bases 390 and 391 (fig. 3). Although this does not result in a directly mismatched T in either sense strand of the probe, both strands of the probe have a T immediately adjacent to the insertion. The presence of the insert would sufficiently disrupt pairing of both these thymines to allow reaction with OsO_4 (Cotton and Campbell 1989) (fig. 4). The triplet ACT codes for insertion of an extra threonine between alanine 122 and threonine 123.

The mechanism for the origin of this mutation is not known. When genomic DNA was used as the target for PCR, only the expected 83-bp (from the normal allele) and 86-bp (from the mutant allele) products predicted from the cDNA sequence were detected (fig. 5). This indicates that the mutation is completely within an exon and that it is not the result of incorrect processing at an intron/exon boundary. Unequal crossing-over during replication is one potential mechanism for this mutation. Clearly this has not occurred precisely at the threonine codon, as the insertion is ACT rather than ACC. However, crossing-over at the second base of the preceding alanine codon, along with subsequent duplication of the bases CTA, could have resulted in the insertion of the extra threonine codon (fig. 3).

A reason for the choice of patient KA for the present study was that the parents were consanguineous and showed the expected heterozygote levels of DHPR activity, as had a younger brother, and that RFLP analysis had shown that the patient was homozygous for the mutant allele (Firgaira et al. 1983; Dahl et al. 1988). Therefore we expected to find only one mutation in the DHPR gene. Both sequence analysis and CCM demonstrated the presence of only the ACT insert and a neutral polymorphism. To confirm that the ACT insertion had the same pattern of inheritance as the informative RFLP pattern (Dahl et al. 1988), PCR was used to amplify the mutant (86-bp) and normal (83-bp) alleles in genomic DNA from the patient KA, the father FA, the mother MA, and the brother BA. Figure 5 shows that both parents and the brother have both mutant and normal alleles, while the patient has only the 86-bp mutant allele. This confirms that the insert segregates with the mutant allele and, combined with the presence of mutant protein, strongly suggests that this is indeed the causative mutation in this child.

Precisely how this mutation inactivates DHPR is not known. Under denaturing conditions the protein has the same electrophoretic and immunological properties as normal (Firgaira et al. 1981*a*, 1981*b*), but these properties would not necessarily change on addition of an extra threonine. The mutation might disrupt protein folding, causing loss of the pteridine or nicotinamide cofactor-binding site, or might prevent dimer formation. Alternatively, the mutation might result in a

more subtle alteration at the active site. Some support for this latter suggestion comes from the finding that the threonine insert lies close to a small region of homology, centered around Cys^{104} in hDHPR and Gly^{20} in human dihydrofolate reductase (Dahl et al. 1987; Lockyer et al. 1987), which has been implicated in the binding of the nicotinamide cofactor to dihydrofolate reductase (Lai et al. 1982).

Detailed analysis of this mutant protein, expressed in bacteria, will allow us to determine how this small change alters the activity of the enzyme and whether it occurs at the active site. Similar analysis of further patients might allow us to build a picture of which amino acids form the active site.

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