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Human dihydropteridine reductase: characterisation of a cDNA clone and its use in analysis of patients with dihydropteridine reductase deficiency

H.-H.M.Dahl*, W.Hutchison, W.McAdam, S.Wake, F.J.Morgan[1] and R.G.H.Cotton

Murdoch Institute for Research into Birth Defects, Royal Children's Hospital, Melbourne 3052 and [1]St. Vincent's Institute of Medical Research, Melbourne 3065, Victoria, Australia

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ABSTRACT

Deficiency of human dihydropteridine reductase (hDHPR) causes malignant hyperphenylalaninemia. We report the isolation of a cDNA clone for hDHPR that spans the complete coding region, and present the nucleotide sequence and the predicted amino acid sequence. The hDHPR protein does not share extensive homology with the enzymatically related protein human dihydrofolate reductase. Patients with hDHPR deficiency were analysed for the presence of hDHPR cross-reacting protein, mRNA encoding hDHPR, and chromosomal DNA rearrangements. The results show that this inherited error of metabolism can result from a variety of mutations. However, no major rearrangements were seen in 11 patients analysed by Southern blotting. Three RFLPs were found with the restriction endonucleases AvaII and MspI. These RFLPs are useful for prenatal diagnosis of hDHPR deficiency.

INTRODUCTION

Human dihydropteridine reductase (hDHPR, EC 1.6.99.7) is the enzyme responsible for catalysing the conversion of quininoid 7,8-dihydrobiopterin to tetrahydrobiopterin (BH_4), an essential cofactor for phenylalanine, tyrosine and tryptophan hydroxylases (1). hDHPR deficiency leads to hyperphenylalaninemia and neurological disturbances (malignant hyperphenylalaninemia), and is generally lethal if untreated (2). Initially the interest in hDHPR was mainly due to its importance in phenylketonuria, but more recently lack of BH_4 has been implicated in other neurological diseases (38). It has been shown that in 1-3% of patients with phenylketonuria the enzyme defect is in the synthesis or recycling of BH_4 rather than in phenylalanine hydroxylase. Deficiency of hDHPR is the cause of the disease in about 30% of these cases (37). It is important that these patients are diagnosed correctly at an early stage, as the treatment and prognosis differ from those of classical phenylketonuria (2-9, 36).

hDHPR deficiency was the first of the inborn errors of tetrahydrobiopterin metabolism to be described (10,40) and it is inherited as an autosomal recessive trait. hDHPR activity has been detected in all tissues and cell types tested which suggests that the enzyme is also of importance for metabolic pathways other than oxidation of the aromatic amino acids.

Human liver DHPR has been purified to homogeneity (11) and is similar to the corresponding sheep and bovine enzymes with respect to molecular weight and amino acid composition. The properties of this enzyme have recently been reviewed (12, 36).

To further analyse the structure and expression of hDHPR we have isolated cDNA clones containing the complete coding region, and have determined the nucleotide sequence. The cDNA clone has been used to probe RNA blots from normal individuals and patients with hDHPR deficiency and the results indicate that the mutations causing hDHPR deficiency are heterogeneous in nature. Finally, we describe 3 RFLPs, detected with the restriction enzymes AvaII and MspI, which will be useful in prenatal diagnosis.

MATERIALS AND METHODS

Purification of Human DHPR

Dihydropteridine reductase was purified from human liver essentially as described previously (11). An additional step was included after extraction of the liver tissue: the pH of the liver extract was lowered to pH 4.0 with HCl,and the extract centrifuged at 15,000 g for 30 min. The pH of the supernatant was increased to pH 7.6, and after centrifugation as above, the supernatant was passed through the napthoquinone column. CM-Sephadex chromatography was omitted. The enzyme was approximately 98% pure based on densitometric scans of stained patterns obtained from analytical SDS-polyacrylamide gel electrophoresis (13).

Antibody Production

An antiserum was raised in a rabbit against purified human liver dihydropteridine reductase (14). This antiserum was purified by affinity chromatography using an hDHPR-Sepharose 4B column (made by binding 1 mg purified hDHPR to 0.5 ml CNBr activated Sepharose 4B [Pharmacia]). The antibody was eluted from the immuno-affinity column using 0.1 M glycine-HCl pH 2.5 and the eluate neutralised immediately. The purified antibody was analysed by immunoblotting. Human liver extract (300µg) was separated on

SDS-polyacrylamide gels and transferred to nitrocellulose as described (15). The nitrocellulose was incubated with antiserum (diluted 1/500 in 10 mM Tris-HCl pH 7.4; 0.15 M NaCl 3% w/v serum albumin). After washing, bound antibody was visualised using an alkaline-phosphatase conjugated anti-rabbit antibody and developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Promega).

Peptide Purification

Carboxamidomethylation of purified enzyme was performed in 6M guanidine hydrochloride, 2mM EDTA, 0.2M Tris-HCl, pH 9.0 as described (16). Trypsin digestion of the treated protein was performed at 37°C for 6 hours with a weight ratio of proteinase to substrate of 1:50. DHPR tryptic peptides (200 µg) were separated on a Brownlee Aquapore RP300 column (10 µm; 4.6mm x 25cm). The column was equilibrated in 0.1% TFA and the peptides were eluted by a linear gradient of acetonitrile from 8 to 60% over a period of 40 mins. The UV profile was observed at 215nm. Cyanogen bromide digestion of DHPR was performed in 70% formic acid at 25°C for 6 hours. Peptides were separated by HPLC as above. Selected peptides were subjected to automated Edman degradation and PTH amino acids were analysed as described (17).

Isolation of hDHPR cDNA Clones

A human fetal liver cDNA library constructed in the expression vector λgt11 (supplied by Clontech Inc., # HL1055) was plated on E.coli RY1090 (18). Approximately 70,000 IPTG-induced recombinant plaques were screened with the affinity-purified antibody to hDHPR using the Promega Protoblot Immunoscreening system. The cDNA inserts were separated from the λgt11 DNA after digestion with EcoRI and subcloned into Bluescribe M13- (Stratagene, Calif.). The cDNA insert from one of these clones (λhDHPR 5) was used to screen approximately 80,000 recombinant plaques from a human liver cDNA-λgt11 library (Clontech Inc., # HL1001b) following the method of Benton and Davis (19).

Oligonucleotide probes based on the peptide sequence data (later shown to correspond to amino acid 155-167 and 170-176 in Fig. 1) were synthesised on an Applied Biosystems oligonucleotide synthesiser. Probes were labelled with ^{32}P using polynucleotide kinase and used for screening, essentially as described by Wallace et.al. (20). A probe complementary to nucleotides 89-107 (Fig. 1) was synthesised and used as described above. It was also used as a primer in M13 DNA sequencing similar to the commonly used M13 DNA sequencing primers.

DNA and RNA Analysis

DNA was sequenced using the M13-dideoxy sequencing method (21). M13 clones were generated by subcloning restriction fragments or size-selected sonicated cDNA fragments into M13mp8, mp18 or mp19 (22). RNA was isolated from cultured human fibroblasts or transformed lymphocytes by the method of Wake and Mercer (23) and RNA blot analysis carried out as previously described (24). DNA was isolated from human blood (white cell pellets) or cultured human fibroblasts according to the method of Weeks et.al. (25). After centrifugation for 5 hrs. at 100,000 rpm in a Beckman TLA-100.2 rotor, the chromosomal DNA band was ethanol-precipitated and resuspended in 10 mM Tris-HCl (pH 7.5), 1mM EDTA. Southern blot analysis involved transfer onto Gene Screen Plus membranes (New England Nuclear). The blots were hybridised at 42°C with ^{32}P-labelled probes (labelled by oligonucleotide priming [26]) in accordance with the manufacturer's protocol. Enzymes were purchased from Boehringer Mannheim.

RESULTS

Characterisation of Antibodies

The specificity of the antiserum before and after immuno-affinity purification was tested by immunoblotting with human liver extract. The crude antiserum reacted with a number of proteins in the liver extract. However, following the affinity purification of the antibody, only a single band - corresponding to the hDHPR subunit - was detected (results not shown).

Isolation and Characterisation of cDNA Clones

Screening the amplified human fetal liver cDNA-λgt11 library with the affinity purified antibody to hDHPR resulted in the isolation of 7 clones (λ hDHPR 1-7) that were positive on rescreening. DNA was purified from these 7 clones and the size of the cDNA inserts estimated by agarose gel electrophoresis of EcoRI digests of the DNAs. λhDHPR 5 appeared to have the largest insert (approximately 1200 bp). These cDNAs also hybridised to the degenerate oligonucleotide probes (results not shown), whose sequences were based on peptide sequence data (Table I). DNA sequence analysis of clone λDHPR 5 showed that its 5' end mapped at nucleotide 28 (Fig. 1). An open reading frame was present in the cDNA and the deduced protein sequence contained the tryptic and CNBr peptides determined by protein sequencing (Table 1). However, no initiator methionine was present and we therefore screened the human adult liver cDNA library (in λgt11) using the ^{32}P

TABLE 1

Amino acid sequence of trypsin and CNBr derived hDHPR peptides (determined as described in Materials and Methods). Their location in the predicted amino acid sequence for hDHPR (Fig. 1) is also shown.

Tryptic peptides	Amino acid no. in Fig. 1
G A L G S R	20-25
V L V Y G G R	13-19
G A V H Q L C Q S L A G K	155-167
S L F K	99-102
N S G M P P G A A A I A V L P V T L D	168-186
CNBr-peptides	
M I G Y	147-150
M A K G A V H Q L C Q S L A G K N	152-168
M P P G A	171-175

labelled cDNA insert from λ hDHPR 5. This led to the isolation of 19 new clones, 5 of which hybridised to a unique oligonucleotide probe complementary to nucleotides 89-107 (Fig. 1). The sequence of one of these, λhDHPR 19 is shown in Fig. 1. Also shown is a restriction enzyme map of this clone (Fig. 2). It has an ATG codon at nucleotide position 25, an open reading frame until nucleotide 757, a 3' non-coding region of 461 bp and a poly(A) tail. A variant (ATTAAA) of the common polyadenylation signal is located 30 bp upstream from the poly(A) site. This variant has been noted in other cDNA clones (27-30). The 5' untranslated cDNA region in clone λDHPR 19 is highly G rich (54%). The 5' end of the coding region is GC rich (75% GC in nucleotides 25-120). However, this high GC content is not seen in the rest of the coding region and the 3' untranslated region is slightly AT rich.

hDHPR Protein

A protein sequence has been deduced from the cDNA sequence presented in Fig. 1. The peptide sequences obtained for purified tryptic and CNBr fragments are found in the predicted amino acid sequence (Table 1). Furthermore, the deduced amino acid composition agrees well with published data for this enzyme (Table 2). The lower content of methionine, cysteine and tryptophan quoted in ref. 11, compared to the deduced composition, is likely be due to oxidation of the protein sample during purification and analysis. The amino acid composition of the human DHPR is very similar to that of the bovine and sheep enzymes, and presumably reflects their high

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                                                                10
                                MetAlaAlaAlaAlaAlaGlyGluAlaArgArgValLeuValTyrGlyGlyArgGlyAlaLeu
CGGAGCCGGGCTGGCAGGAGCAGGATGGCGGCGGCGGCGGCTGCAGGCGAGGCCGCGCCGGGTGCTGGTGTACGGCGGCAGGGGCGCTCTG    90

                        30                              40
GlySerArgCysValGlnAlaPheArgAlaArgAsnTrpTrpValAlaSerValAspValValGluAsnGluGluAlaSerAlaSerIle
GGTTCTCGATGCGTGCAGGCTTTTCGGGCCCGCAACTGGTGGGTTGCCAGCGTTGATGTGGTGGAGAATGAAGAGGCCAGCGCTAGCATC   180

                        60                              70
IleValLysMetThrAspSerPheThrGluGlnAlaAspGlnValThrAlaGluValGlyLysLeuLeuGlyGluGluLysValAspAla
ATTGTTAAAATGACAGACTCGTTCACTGAGCAGGCTGACCAGGTGACTGCTGAGGTTGGAAAGCTCTTGGGTGAAGAGAAGGTGGATGCA   270

                        90                              100
IleLeuCysValAlaGlyGlyTrpAlaGlyGlyAsnAlaLysSerLysSerLeuPheLysAsnCysAspLeuMetTrpLysGlnSerIle
ATTCTTTGCGTTGCTGGAGGATGGGCCGGGGGCAATGCCAAATCCAAGTCTCTCTTTAAGAACTGTGACCTGATGTGGAAGCAGAGCATA   360

                        120                             130
TrpThrSerThrIleSerSerHisLeuAlaThrLysHisLeuLysGluGlyGlyLeuLeuThrLeuAlaGlyAlaLysAlaAlaLeuAsp
TGGACATCGACCATCTCCAGCCATCTGGCTACCAAGCATCTCAAGGAAGGAGGCCTCCTGACCTTGGCTGGCGCAAAGGCTGCCCTGGAT   450

                        150                             160
GlyThrProGlyMetIleGlyTyrGlyMetAlaLysGlyAlaValHisGlnLeuCysGlnSerLeuAlaGlyLysAsnSerGlyMetPro
GGGACTCCTGGTATGATCGGGTACGGCATGGCCAAGGGTGCTGTTCACCAGCTCTGCCAGAGCCTGGCTGGGAAGAACAGCGGCATGCCG   540

                        180                             190
ProGlyAlaAlaAlaIleAlaValLeuProValThrLeuAspThrProMetAsnArgLysSerMetProGluAlaAspPheSerSerTrp
CCCGGGGCAGCCGCCATCGCTGTGCTCCCGGTTACCCTGGATACCCCGATGAACAGGAAATCAATGCCTGAGGCTGACTTCAGCTCCTGG   630

                        210                             220
ThrProLeuGluPheLeuValGluThrPheHisAspTrpIleThrGlyLysAsnArgProSerSerGlySerLeuIleGlnValValThr
ACACCCTTAGAATTCCTAGTTGAAACTTTCCATGACTGGATCACAGGGAAAAACCGACCGAGCTCAGGAAGCCTAATCCAGGTGGTAACC   720

                        240
ThrGluGlyArgThrGluLeuThrProAlaTyrPhe***
ACAGAAGGAAGGACCGGAACTCACCCCAGCATATTTTTAGGCCTCATCTCAGTGCCTATGAGGGGCCTGCCAGAAAAGTCACTAACCTGTC   810

TCAGTGTGGCCTTGTCCAGCCTTGTGTTTTCTGTAACCCCTGTTTGTGGTACGAGATAATGAGTCCTATTTTTCTCTCACATAATATGCA   900

TTTGCTCTCCTAGGACAGTGTAATACATTTATGTGAAGTAAAGACATGCGAGACTGGTGGCCTGCAAATAGCATCCGTCAATCTGTGTTA   990

ACTGCATAGGGAGGGCTCTGCATAGCACCTGCTATAGCGGTGTCATGTTGGATCGCTTTTGTGACTGTTCATCTGTCCTTGACAGTGGCT  1000

GTCATCTTGACTACTTTGTTGATTTGTTGGTATTGGGGACATTTTAAAGGCTGAGTTATTTTTGAATGTCATGTTTATGTCATAGACGTA  1170

GTTTTCGCATCCTTGAATTAAACTGCCTTAACTCCTTTTGTGGTATAAAAAAAAAAAAAAAAAAAAAA                        1239
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Figure 1. Nucleotide sequence of the hDHPR cDNA insert from clone λhDHPR 19, and the predicted amino acid sequence of the protein. The nucleotide numbers are at the end of each line, and the amino acids are numbered from the putative translation initiation methionine.

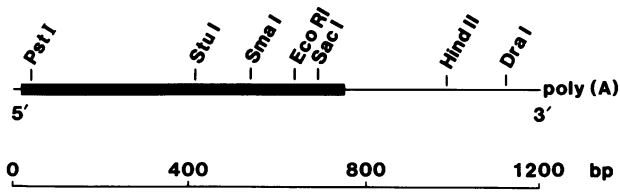

Figure 2. Restriction enzyme map of the λDHPR 19 cDNA, derived from the nucleotide sequence in Fig. 1. The coding region is shown as a solid box.

degree of homology. Attempts by ourselves and others to obtain amino acid sequence data from the N-terminal end of the mature enzyme have been unsuccessful as it appears that the N-terminal amino acid is blocked by

TABLE 2

Amino acid composition of hDHPR deduced from the cDNA sequence and from amino acid analysis (ref. 11).

Amino acid	hDHPR (this study)	hDHPR (ref. 11)
Aspartic acid	9	16
Asparagine	7	
Threonine	17	17
Serine	19	18
Glutamic acid	14	22
Glutamine	7	
Proline	9	9
Glycine	25	26
Alanine	33	31
Valine	18	17
Methionine	8	4
Isoleucine	9	8
Leucine	21	21
Tyrosine	3	3
Phenylalanine	7	7
Histidine	4	5
Lysine	14	14
Arginine	9	9
Cysteine	4	3
Tryptophan	7	5

acetylation (31). The molecular weight of the deduced hDHPR precursor is 25,760, which is close to the figure of 26,000 for the mature protein as determined by SDS-gel electrophoresis (11).

Analysis of Patients with hDHPR Deficiency

Fibroblast or transformed lymphocyte cell lines from patients with hDHPR deficiency have previously been analysed for antibody cross-reacting material (CRM) (9,41,42). The data showed that the molecular defects at the protein level were heterogeneous. We have now extended these studies to include analysis of the mRNA by RNA blotting and of the gene structure by Southern blotting. Fig. 3 shows CRM data and the result of RNA analysis for 12 patients and 2 unaffected controls. In the control RNA samples our hDHPR cDNA probe detected an RNA of approximately 1500 bp. RNA of a similar size was detected in 11 of the patient cell lines. However, in patient cell line 17a, little or no mRNA was seen, suggesting defect(s) affecting transcription and/or mRNA stability in both hDHPR alleles. The smear seen in the RNA from patient cell line MK did not appear to be caused by degradation of the total RNA as the 28S and 18S ribosomal RNA bands did not

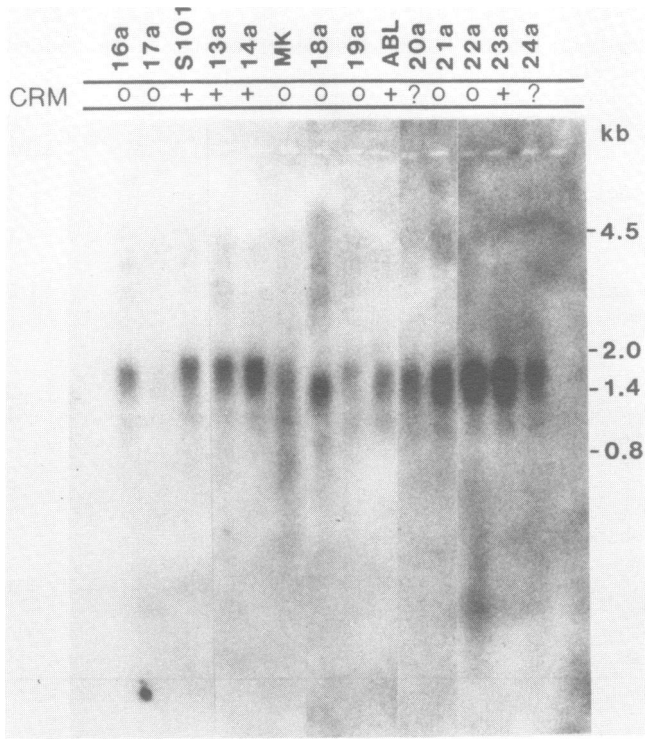

Figure 3. CRM and RNA blot analysis of patients with hDHPR deficiency. RNA was isolated from fibroblast (13a, 14a, 16a-24a, S101) or transformed lymphocyte cell lines (ABL, MK). Approximately 10 µg total RNA was electrophoresed, blotted and hybridised to the hDHPR cDNA probe. Cell lines S101 and ABl are normal controls, and the rest are from individuals with hDHPR deficiency. Also shown is the hDHPR CRM status of the cell lines, determined as described in ref. 9. + and 0 indicates the presence or absence of cross-reacting material, and ? indicates that the CRM status is not known.

show degradation. The result therefore suggests that the hDHPR mRNA in this patient is unusually unstable.

Chromosomal DNA from 11 patient cell lines and a normal control cell line was analysed by Southern blotting after digestion with restriction endonuclease BamHI or Hind III. No rearrangements or total gene deletions were detected (results not shown).

AvaII and MspI show RFLPs, Useful in Prenatal Diagnosis

In order to find RFLPs for prenatal diagnosis of hDHPR deficiency, chromosomal DNA from normal individuals was analysed by Southern blotting. The following restriction endonucleases were tested: AvaII, BamHI, BclI, EcoRI, EcoRV, HindIII, MspI and TaqI. RFLPs were found with the enzymes

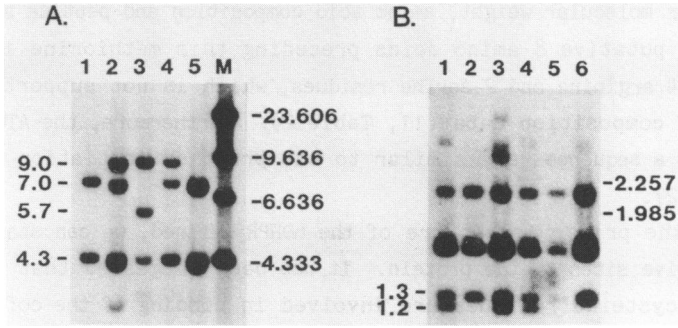

Figure 4. DNAs showing the Southern blot analysis of human RFLPs detected with restriction enzymes AvaII and MspI. Approximately 5 μg DNA from normal individuals was digested with the restriction enzyme and the blot hybridised to the hDHPR cDNA probe. Panel A shows the AvaII polymorphisms. Lanes 1-5 are the human DNAs digested with AvaII, lane M is a λ-HindIII marker. The sizes of the polymorphic bands are indicated to the left of the panel. Panel B shows the MspI polymorphism. Lanes 1-6 are human DNAs digested with MspI. The sizes of the polymorphic bands are indicated to the left, and molecular weight marker bands to the right of the panel.

AvaII and MspI (Fig. 4). Two RFLPs were detected with AvaII: one created two allelic fragments of 9.0 and 7.0 kb, and the other created fragments of 5.7 and 4.3 kb. The frequencies of the alleles observed in 24 unrelated caucasians were 0.62 (9.0 kb), 0.38 (7.0 kb), and 0.23 (5.7 kb), 0.77 (4.3 kb). MspI-digested human DNA showed a two-allele RFLP with bands at 1.3 kb and 1.2 kb. The frequencies of these RFLPs in 25 unrelated caucasians were 0.74 (1.3 kb) and 0.26 (1.2 kb). The calculated heterozygote frequencies for the 3 polymorphisms are 0.47, 0.35, and 0.38 respectively, which suggests that these RFLPs will be useful in prenatal diagnosis.

DISCUSSION

We have isolated a hDHPR cDNA clone that appears to contain the entire coding region for this enzyme. The evidence that this is a genuine hDHPR clone is the following: when expressed in λgt11 the fusion protein reacts strongly with an affinity purified anti-hDHPR antibody; the amino acid composition and molecular weight agree well with published data; and tryptic and CNBr peptide sequences (obtained from purified hDHPR) are found in the predicted amino acid sequence. The length of the cDNA clone is 1239 bp and it comprises a 24 bp 5' non-translated region, a 732 bp coding region, and a 461 bp 3' non-coding segment, followed by a poly(A) tail (Fig. 1).

From the cDNA sequence a protein sequence has been deduced. We suggest that the methionine of nucleotide 25 is the initiator methionine. This fits

the data for molecular weight, amino acid composition and peptide sequences. Also, the putative 8 amino acids preceding this methionine include an additional 4 arginine and 2 serine residues, which is not supported by the amino acid composition data (11, Table 2). Furthermore, the ATG codon is preceded by a sequence very similar to the proposed initiation consensus sequence (32).

With the primary structure of the hDHPR defined, we can start to look for the active sites of the protein. It has been suggested that the thiol groups of cysteine residues are involved in binding of the cofactor NADH (33). The predicted hDHPR sequence contains 4 cysteine residues which are situated in the middle or towards the N-terminal end of the protein.

More information on the active sites might come from comparison of hDHPR with the related enzyme human dihydrofolate reductase (hDHFR), which also has pterin and nicotinamide derived cofactors as substrates. A computer comparison of the protein sequences using the DIAGON program (34) did not reveal extensive similarity. However, a small region of homology centred around Cys^{104} in hDHPR and the corresponding Gly^{20} in hDHFR (35) was observed.

	104								
hDHPR	Leu	Phe	Lys	Asn	Cys	Asp	Leu	Met	Trp	Lys
hDHFR	Ile	Gly	Lys	Asn	Gly	Asp	Leu	Pro	Trp	Pro
					20					

This region of the hDHFR protein, and especially Trp^{24}, has been implicated in the binding of nicotinamide (39), and might have a similar function in hDHPR. However, further studies are needed to define the active sites in hDHPR.

A computer assisted search of the databases Newat (Doolittle), NBRF PIR, PSD-Kyoto (Ooi/Nakashima), and PG trans (compiled from GenBank) did not reveal significant homologies between the hDHPR amino acid sequence and other published protein sequences.

Patients with hDHPR deficiency were examined for the presence of antibody cross-reacting material and mRNA (Fig. 3). In patient 17a neither protein nor mRNA was detected. In patients 16a, 18a, 19a, 21a and 22a mRNA, but no protein, was seen. In patients 13a, 14a and 23a mRNA and CRMwas detected, but the enzyme was inactive. The broad RNA band seen in case MK indicates that the hDHPR mRNA in this patient is unstable. These results therefore suggest that a variety of mutations are causing the defects in hDHPR expression. Southern blot analysis of DNA from 11 patients with hDHPR

deficiency did not reveal major rearrangements in or around the hDHPR gene, and suggests that deletions are not a frequent cause of hDHPR deficiency.

Two restriction enzymes (AvaII and MspI) were shown to give useful RFLPs. This has implications for prenatal diagnosis of hDHPR deficiency. Enzyme assays of hDHPR activity are available for prenatal diagnosis (reviewed in ref. 12). However, as with most enzyme assays of inborn errrors of metabolism they require considerable expertise and special reagents and equipment. The blotting technique required for detection of RFLPs is universally available and reproducible, and is therefore more likely to be of general use. As more restiction enzymes are tested the number of RFLPs should increase and thereby improve the percentage of informative families.

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*To whom correspondence should be addressed

REFERENCES

1. Kaufman, S. and Fisher, D.B. (1974) In Molecular Mechanism of Oxygen Activation, Hayaishi, O. (ed.), Academic Press, New York pp.285-369.
2. Danks, D.M., Bartholome, K., Clayton, B.E., Curtius, H., Gröbe, H., Kaufman, S., Leeming, R., Pfleiderer, W., Rembold, H., and Ray, F. (1978) J. Inher. Metab. Dis. **1**, 49-54.
3. Kaufman, S. (1985) J. Inher. Metab. Dis. **8** Suppl. 1, 20-27.
4. Bartholome, K., Byrd, D.J., Kaufman, S. and Milstien, S. (1977) Pediatrics **59**, 757-761.
5. Butler, I.J., O'Flynn, M.E., Seifert, Jr., W.E. and Howell, R.R. (1981) J. Pediatr. **98**, 729-733.
6. Niederwieser, A., Curtius, H.-Ch., Viscontini, M., Schaub, J. and Schmidt, H. (1979) Lancet **1**, 550.
7. Danks, D.M., Cotton, R.G.H. and Schlesinger, P. (1975) Lancet **2**,1043.
8. O'Brien, D., Berlow, S., Donnell, G., Justice, P., Kaufman, S., Levy, H.L., McCabe, E.R.B. and Snyderman, S. (1980) Pediatrics **65**, 844-846.
9. Cotton, R.G.H., Jennings, I., Bracco, G., Ponzone, A. and Guardamagna, O. (1987) J. Inh. Metab. Dis. (in press).

10. Smith, I., Clayton, B.E., and Wolff, O.H. (1975) Lancet **1**, 1108-1111.
11. Firgaira, F.A., Cotton, R.G.H. and Danks, D.M. (1981) Biochem. J. **197**, 31-43.
12. Armarego, W.L.F., Randles, D., and Waring, P. (1984) Medicinal Research Reviews **4**, 267-321.
13. Laemli, V.K. (1970) Nature **227**, 680-685.
14. Firgaira, F.A., Choo, K.H., Cotton, R.G.H. and Danks, D.M. (1981) Biochem. J. **197**, 45-53.
15. Towbin, H., Stehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350-4354.
16. Mercer, J.F.B., McAdam, W., Chambers, W. and Walker, I.D. (1986) **236**, 679-683.
17. Simpson, R.J., Begg, G.S., Dorow, D.S. and Morgan, F.J. (1980) Biochem. **19**, 1814-1819.
18. Huyhn, T.V., Young, R.A. and Davis, R.W. (1985) In Glover, D. (ed.) DNA Cloning Techniques: A Practical Approach, IRL Press, Oxford, pp.49-78.
19. Benton, W.D. and Davis, R.W. (1977) Science **196**, 180-182.
20. Wallace, R.B., Johnson, M.J. Hirose, T., Miyake, T., Kawashima, E.H.and Itakura, K. (1981) Nucl. Acids Res. **9**, 879-894.
21. Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 5463-5467.
22. Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) Gene **33**, 103-119.
23. Wake, S.A. and Mercer, J.F.B. (1985) Biochem. J. **228**, 425-432.
24. Dahl, H.-H.M. and Mercer, J.F.B. (1986) J. Biol. Chem. 261, 4148-4153.
25. Weeks, D.P., Beerman, N. and Griffith, O.M. (1986) Anal. Biochem. **152**, 376-385.
26. Feinberg, A.P. and Vogelstein, B. (1983) Anal. Biochem. **132**, 6-13.
27. Swimmer, C. and Shenk, T. (1985) Nucl. Acids Res. **13**, 8053-8063.
28. Jung, A., Sippel, A.E. Grez, M. and Schutz, G. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 5759-5763.
29. Fornwald, J., Kuncio, G., Peng, I. and Ordahl, C.P. (1982) Nucl. Acids Res. **10**, 3861-3876.
30. Ahmed, G.M., Chanda, R., Stow, N. and Zain, B.S. (1982) Gene **19**, 297-301.
31. Korri, K.K., Chippel, D., Chauvin, M.M., Tirpak, A. and Scrimgeour, K.G. (1977) Can. J. Biochem. **55**, 1145-1152.
32. Kozak, M. (1984) Nucl. Acids Res. **12**, 857-872.
33. Webber, S. and Whiteley, J.M. (1981) Arch. Biochem. Biophys. **206**, 145-152.
34. Staden, R. (1982) Nucl. Acids Res. **10**, 2951-2961.
35. Masters, J.N. and Attardi, G. (1983) Gene **21**, 59-63.
36. Kaufman, S. (1986) J. Pediat. **109**, 572-578.
37. Niederwieser, A., Ponzone, A. and Curtius, H.-Ch. (1985) J. Inher. Metab. Dis. **8**, Suppl. 1, 35-38.
38. Cotton, R.G.H. In Blakley, R.L. and Whitehead, V.M. (eds) The Biochemistry of Folic Acid and Related Pteridines, Elsevier, Amsterdam, Vol.2 (in press).
39. Lai, P.-H., Pan. Y.-C.E., Gleisner, J.M., Peterson, D.L., Williams, K.R., and Blakley, R.L. (1982) Biochemistry **21**, 3284-3294.
40. Kaufman, S., Holtzman, N.A., Milstein, S., Butler, I.J. and Krumholz, A. (1975) New Engl. J. Med. **293**, 785-790.
41. Firgaira, F.A., Choo, K.H., Cotton, R.G.H. and Danks, D.M. (1981) Biochem.J. **198**, 677-682.
42. Firgaira, F.A., Cotton, R.G.H. and Danks, D.M. (1983) in Blair, J.A. (ed.) Chemistry and Biology of Pteridines, W. de Gryter, Berlin, pp. 771-775.