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Dihydropyrimidinase Deficiency: Structural Organization, Chromosomal Localization, and Mutation Analysis of the Human Dihydropyrimidinase Gene

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Summary

Dihydropyrimidinase (DHP) deficiency (MIM 222748) is characterized by dihydropyrimidinuria and is associated with a variable clinical phenotype. This disease might be associated with a risk of 5-fluorouracil toxicity, although no cases have been reported. We present here both the molecular characterization of the human DHP gene and, for the first time, the mutations causing DHP deficiency. The human DHP gene spans >80 kb and consists of 10 exons. It has been assigned to 8q22, by FISH. We performed mutation analysis of genomic DNA in one symptomatic and five asymptomatic individuals presenting with dihydropyrimidinuria. We identified one frameshift mutation and five missense mutations. Two related Japanese adult subjects were homozygous for the Q334R substitution, whereas two other, unrelated Japanese infant subjects were heterozygous for the same mutation, but this mutation is not common in the Japanese population. A Caucasian pediatric patient exhibiting epileptic attacks, dysmorphic features, and severe developmental delay was homozygous for W360R. Using a eukaryotic expression system, we showed that all mutations reduced enzyme activity significantly, indicating that these are crucial DHP deficiency–causing mutations. There was no significant difference, in residual activity, between mutations observed in the symptomatic and those observed in the asymptomatic individuals.

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Introduction

Dihydropyrimidinase (DHP [E.C.3.5.2.2]) is the second enzyme in the three-step degradation pathway of uracil and thymine. DHP catalyzes the reversible hydrolysis of dihydrouracil to N-carbamyl-β-alanine and of dihydrothymine to N-carbamyl-β-aminoisobutyric acid (Fritzson 1957; Fritzson and Phil 1957). DHP is a homotetrameric metalloenzyme, containing four tightly bound zinc ions per molecule of active enzyme (Brooks et al. 1979, 1983; Kikugawa et al. 1994). Purified DHP from rat liver has a molecular mass of 215 kD and a subunit mass of 54 kD (Kikugawa et al. 1994). We isolated cDNA clones encoding DHP from human and rat liver, which contained 1,560-bp open reading frames encoding polypeptides of 519 residues (Hamajima et al. 1996; Matsuda et al. 1996). Moreover, we isolated three homologous cDNA clones from human fetal brain, which we named "DHP related proteins" (DRPs) and thus identified a novel gene family defined by DHP and DRPs with differential tissue distribution (Hamajima et al. 1996).

DHP deficiency (MIM 222748) is thought to be an autosomal recessive disorder that is characterized by dihydropyrimidinuria and associated with a variable clinical phenotype. DHP deficiency might be associated with the pharmacogenetic syndrome of 5-fluorouracil (5-FU) toxicity (Hayashi et al. 1996), although no cases have been reported. To date, nine cases of dihydropyrimidinuria have been reported. Four unrelated cases showed variable clinical symptoms: epileptic or convulsive attacks (Duran et al. 1990; Henderson et al. 1993; Van Gennip et al. 1997*b*), dysmorphic features and severe developmental delay (Henderson et al. 1993; Van Gennip et al. 1997*b*), and congenital microvillous atrophy (Assmann et al. 1997). However, the relationship between these symptoms and the enzyme defect has not

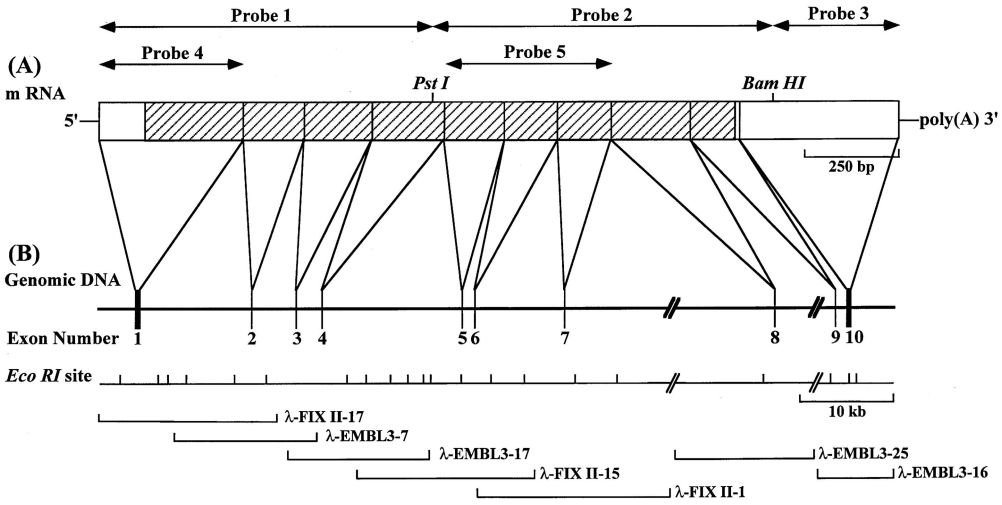

Figure 1 Schematic representation of genomic and mRNA structure of human DHP. *A*, Structure of human DHP mRNA (Hamajima et al. 1996). Diagonally hatched and unmarked boxes represent coding and noncoding regions, respectively, arrows indicate the probe-covering regions. *B*, Structure and restriction map of human DHP gene. Exons are denoted by thick vertical rules (exons 1 and 10) and thin vertical lines (exon 2–9) on the "Genomic DNA" line, *Eco*RI sites are indicated by the vertical tic marks on the "*Eco*RI site" line, and isolated phage clones containing genomic DNA fragments encoding the human DHP gene are shown below the "*Eco*RI site" line.

been elucidated. On the other hand, three unrelated infant and two related adult cases without clinical symptoms were discovered by a program screening for pyrimidine-degradation disorders in a healthy Japanese population (Ohba et al. 1994; Sumi et al., in press). The population study based on this screening program revealed a relative high frequency of this condition, of 0.1% (2:20,000), in the Japanese population (Sumi et al., in press). In only two cases, DHP deficiency was enzymatically confirmed by direct measurement of liver DHP activity (Assmann et al. 1997; Van Gennip et al. 1997*b*). In the present article we describe both the molecular characterization of the human DHP gene and, for the first time, mutations causing DHP deficiency, which are identified by molecular analysis of genomic DNA in one symptomatic and five asymptomatic individuals presenting with dihydropyrimidinuria.

Subjects, Material, and Methods

Subjects

Asymptomatic subjects 1 and 4 (females) and subject 5 (male) were unrelated and had been born to healthy nonconsanguineous Japanese parents. These subjects were identified at age 6–12 mo, in a program that screened for inborn errors of pyrimidine metabolism and that was operated as a pilot study (Ohba et al. 1994; Sumi et al., in press). Subject 2 was an adult male and had been born to healthy nonconsanguineous Japanese parents. He had no clinical symptoms and had been discovered, at age 37 years, by a screening program (Hayashi et al. 1996; Sumi et al., 1996). Subject 3 was an adult female and a younger sister of subject 2. She had no clinical symptoms and had been discovered, at age 36 years, in a study of the family of subject 2 (Sumi et al., in press). Subject 6 was an infant female born to a consanguineous couple from Lebanon, who exhibits dysmorphic features, severe developmental delay, and convulsions (Van Gennip et al. 1997*b*). In this case, DHP deficiency was confirmed enzymatically, by direct measurement of liver DHP activity. All subjects showed the characteristic biochemical abnormalities observed in DHP deficiency, including elevated concentrations of dihydrouracil, dihydrothymine, uracil, and thymine in their bodily fluids.

Isolation of Genomic Clones

Three DNA fragments, of 878, 903, and 329 bp, were prepared by *Pst*I and *Bam*HI digestion of a plasmid DNA containing the human DHP cDNA and were used as probes 1, 2, and 3, respectively, for screening of a λ-EMBL3 human genomic DNA library (Japanese Cancer Research Resources Bank) (fig. 1). Two DNA fragments, of 367 and 429 bp, were prepared by PCR and were used as probes 4 and 5, respectively, for screening of a λ-FIX II human genomic DNA library (Stratagene) (fig. 1). The latter PCR reactions were performed with the plasmid DNA containing the human DHP cDNA as a template, with sense primer 5′-GGC TGT CGG TGG GGA CCT TG-3′ and antisense primer 5′-GGT GCC CTG GTT GGA AGT TCG T-3′, for probe 4, and with sense primer 5′-GAA GGT GGT CTA TGG TGA AC-

Table 1

PCR Primers to Amplify Exons of the Human DHP Gene

Exon	Primer[a] (5′→3′) Sense	Primer[a] (5′→3′) Antisense	Product Length (bp)
1	TGCAGGAGGGCACCCCAAGC	gaggcggccctgctgaggac	461
2	atgcccttctgggtcattta	tctgtcctcctgttgtgtag	423
3	gagcagcagcagtttatcag	gcccaatcatcttcacctta	454
4	gatcaaaagcctggcattgc	ctcctaaactgaagcagagg	376
5	tatctggtagggttttggag	ggatcctggctgaagaacta	532
6	gagacaggagagggatgaaa	ggatcctggctgaagaacta	362
7	catcctcagatgctctacaa	ctacatcctctatgccaaga	476
8	tcaagtgagctggtgatgat	ggaaatcccgaactgaccta	549
9	cacaaaaagtgggacaatcc	gtgaagcctctgaccttgat	421

[a] Exon and intron sequences are denoted by uppercase and lowercase letters, respectively.

3′ and antisense primer 5′-GGG TCC CAA ATA ACA ATG TC-3′, for probe 5. Probes were labeled with $\alpha[^{32}P]$-dCTP, with the aid of a Rediprime DNA-labeling system (Amersham), and were used to screen ~1×10^6 plaques from each human genomic DNA library. Positive clones were analyzed by restriction-enzyme mapping and Southern blotting. Exon-containing DNA fragments were subcloned into pGEM-3Zf(+) vector and were sequenced by a 373A DNA sequencer (Applied Biosystems), to analyze each exon/intron boundary. Each intron size was determined by PCR, except for introns 7 and 8.

FISH

FISH of 5-bromodeoxyuridine–synchronized human metaphase chromosomes was performed as described elsewhere (Takeda et al. 1993), with use of *Eco*RI fragments of genomic DNA of the human DHP gene as probes. The biotin-labeled DNA probes were hybridized to metaphase spreads under conditions that suppressed signals from repetitive DNA sequences. After fluorochrome detection with FITC-conjugated avidin DCS (Vector Laboratories) and biotin-conjugated goat anti–avidin D antibody (Vector Laboratories), the preparations were counterstained with propidium iodide and were examined by a laser scanning microscope (Zeiss LSM).

Analysis of Genomic DNA

Genomic DNA was prepared from peripheral blood leukocytes of subjects 1–6 and selected members of their families, by phenol-chloroform extraction. PCR primers were designed, by use of exon and intron sequences, to amplify the genomic region containing exons 1–9 (table 1). Exon amplification was performed by the following program: 10 min at 95°C, followed by 30 cycles (for exon 1, 35–40 cycles) of 30 s at 95°C, 1 min at 55°C, and 1 min at 72°C (for exon 1, 1 min at 96°C, 1 min at 64°C, and 1 min at 72°C). The resulting amplification products were subcloned into a pCR2.1 vector (Invitrogen), and at least eight independent clones were sequenced for each exon, by a 373A DNA sequencer (Applied Biosystems). Both strands of each sequence were determined.

PCR-RFLP Analysis

Since the A1001G transition (Q334R) in exon 6 creates a recognition site for *Msp*I (CCAG→CCGG), PCR-RFLP analysis to detect this mutation was performed for family and population studies. Peripheral leukocyte genomic DNA was isolated from 100 unrelated healthy Japanese volunteers and was subjected to the PCR-RFLP analysis. The resulting 362-bp exon 6 amplification products were digested by *Msp*I and were analyzed by electrophoresis in a 2% agarose gel. Mutant alleles were identified on the basis of a digestion pattern showing a 217- and a 145-bp band, whereas wild-type alleles showed only the undigested, 362-bp band.

SSCP

Since the T1078C transition (W360R) in exon 6 causes no change in the restriction-enzyme site, SSCP analysis of exon 6 was performed for family and population studies. Control DNA samples for population studies were obtained from Dutch newborn-screening cards (~95% Caucasian). Amplification of the genomic region containing exon 6 was performed in a 10-μl reaction containing 20–50 ng genomic DNA; 10 mM Tris/HCl (pH 8.3); 50 mM KCl; 2.5 mM $MgCl_2$; 30 ng each of sense primer (5′-CAC CCC ATC ACT CTC GTG T-3′) and antisense primer (5′-GGA TCC TGG CTG AAG AAC TA-3′); 2.5 mM each of dCTP, dTTP, and dGTP

(Pharmacia); 0.25 mM dATP; 0.1 μCi α[^{32}P]-dATP (Amersham); and 1 U *Taq* polymerase (Perkin Elmer). The amplification reaction was performed for 35 cycles (30 s at 95°C, 1 min at 55°C, and 1 min at 72°C). The 225-bp amplification products were diluted 1:20 in 10 mM EDTA, 0.01% SDS and subsequently were diluted 1:4 in layer mix (2 mM EDTA in formamide). Samples were denatured for 3 min at 94°C and were cooled, at the rate of 1°C/s, to 4°C. Finally, the samples were analyzed on a 8% polyacrylamide (mono:bis 49:1), 5% glycerol gel running for 16 h at 8 W (at room temperature).

Expression-Plasmid Construction and Transfection

An expression plasmid containing the wild-type human DHP cDNA (pCR3-hDHP-WT) was constructed by subcloning the insert of a plasmid containing the complete coding region of the human DHP cDNA into eukaryotic expression vector pCR3 (Invitrogen). Expression plasmids containing the mutant human DHP cDNA (pCR3-hDHP-T68R, pCR3-hDHP-InsA, pCR3-hDHP-Q334R, pCR3-hDHP-W360R, pCR3-hDHP-G435R, and pCR3-hDHP-R490T) were constructed by subcloning the site-directed mutagenesis products for each mutation into pCR3-hDHP-WT. The resulting subclones were analyzed, by sequence analysis, to verify the introduction of the desired mutation and to exclude the presence of artifactually introduced changes. All expression plasmids were purified by means of a plasmid midi kit (QUIAGEN). Transfection into COS-7 cells was performed in 10-cm dishes, with 3 ml of opti-MEM medium (GIBCO-BRL) containing 4 μg of each expression plasmid, 30 μl lipofectamine (2 mg/ml), and 20 μl lipofectamine plus. To normalize for any variability in transfection efficiency, 0.5 μg pSV-βGal (Promega), an expression plasmid containing the *Escherichia coli* β-galactosidase cDNA, was cotransfected as an internal standard. Mock transfection was performed by use of self-ligated pCR3 vector and pSV-βGal.

Enzyme-Activity Assay

Cell extracts were made 24 h after the transfection, by use of 1 × reporter lysis buffer (Promega). The level of exogenous DHP in COS-7 cells transfected with wild-type or mutant expression plasmids was determined by measurement of the decrease of absorbance at 225 nm, by use of 20 mM 5-bromo-dihydrouracil as a substrate and 30 μl cell extracts in 50 mM Tris-HCl (pH 8.2) at 37°C for 30 min, essentially as described by Kikugawa et al. (1994). The level of exogenous β-galactosidase in COS-7 cells transfected with pSV-βGal was determined using a β-galactosidase enzyme assay system (Promega). Protein concentrations were determined by a bicinchoninic acid protein–assay reagent (Pierce), with bovine serum albumin used as a standard. The measurement of absorbance was performed by a DU-640 spectrophotometer (Beckman). The activities in COS-7 cells transfected with the mutant constructs were expressed as the percentage of DHP activity determined in the cells transfected with wild-type expression plasmid after both subtraction of the endogenous activity and normalization by β-galactosidase activity. Statistical analysis was performed by the Mann-Whitney U-test.

Western Blot Analysis

Aliquots of 5–17 μg protein isolated from cell extracts, quantitatively normalized for differences in β-galactosidase activity, were subjected to 10% SDS-PAGE and were transferred to a nitrocellulose filter. Western blot analysis was performed with 1:750-diluted anti–rat liver DHP polyclonal antibody. Protein detection was performed by treatment with 1:3,000-diluted horseradish peroxidase–conjugated goat anti–rabbit IgG antibody (BIO-RAD), followed by staining with HRP color reagent (BIO-RAD).

Northern Blot Analysis

Total RNA from transfected COS-7 cells was extracted by use of ISOGEN (Nippongene). In each lane, 5 μg total RNA was separated on a 1% agarose gel, was blotted onto a Hybond-N nylon membrane (Amersham), and subsequently was hybridized with α[^{32}P]-dCTP–labeled probe 1 (see Isolation of Genomic Clones subsection [above]) and the insert of pSV-βGal, by standard procedures.

Results

Isolation and Characterization of the Human DHP Gene

Screening of the λ-EMBL3 human genomic library with probes 1, 2, and 3 (see Subjects, Material, and Methods section [above]) yielded 4, 1, and 7 positive clones, respectively. These phage clones were analyzed by restriction-enzyme mapping and Southern blotting (fig. 1). To isolate the genomic clones containing the remaining exons, the screening of a λ-FIX II human genomic library with probes 4 and 5 was performed, resulting in 8 and 4 positive clones, respectively (fig. 1). The human DHP gene was found to span >80 kb and contained 10 exons with lengths of 131–420 bp. Nucleotide-sequence data for exon/intron boundaries showed that all introns contained the conserved 5′-GT splice donor and 3′-AG splice acceptor sites (table 2). (The nucleotide-sequence data reported in this article have been submitted to the Genbank database, with accession numbers AB004669–AB004678 for exons 1–10, respectively.) Comparison of the human DHP genomic

Table 2

Exon/Intron Boundaries of the Human DHP Gene

Exon (Size [bp])	cDNA Position of Exon[a]	Donor Site[b]	Intron (Size [kb])	Acceptor Site[b]
1 (393)	−129 to +264	GGGCACCAAG gt acccgccc	1 (12.0)	atgattcc ag GCTGCTCTCT
2 (159)	+265 to +423	GAGTGACCAG gt aaagcacc	2 (5.0)	ttcctttt ag GTTAAAGAAG
3 (180)	+424 to +603	AATTGCAGAG gt acgcattt	3 (3.0)	gtcttttt ag GGAGCAAAGA
4 (190)	+604 to +793	AGGAGAGATG gt aattctcg	4 (15.0)	atctttcc ag GGAAGGTGGT
5 (157)	+794 to +950	TGTTGGCTAA gt aatcattt	5 (1.5)	tcttttac ag TGATGATCTA
6 (142)	+951 to +1092	AAAAGGCGTG gt gggtttca	6 (10.0)	tgctttac ag CATAGTGGTA
7 (143)	+1093 to +1235	AAGGCACAAG gt aagtctaa	7 (>20.0)	acaaatat ag GACTATCTCA
8 (208)	+1236 to +1443	GCGAGACCGG gt gagtgtgc	8 (>5.5)	tctctttc ag ACTTGCACAC
9 (131)	+1444 to +1574	TGTGCCATCG gt aagggact	9 (1.0)	ctcttcaa ag GTAAAAAAAA
10 (420)	+1575 to +1994			

[a] Nucleotides are numbered according to the cDNA sequence, starting with +1 for the first nucleotide of the initiation codon. The transcription initiation site was determined by 5′ rapid amplification of the cDNA end and by ribonuclease-protection assay (data not shown).

[b] Exon and intron sequences are denoted by uppercase and lowercase letters, respectively, and the nucleotide consensus sequence of the introns adjoining the splice junctions is underlined.

sequence with a cDNA sequence reported elsewhere (Hamajima et al. 1996) revealed no differences in the coding region. Southern blot analysis of total human genomic DNA restriction fragments showed that this gene exists as a single copy (data not shown).

Chromosomal Localization of the Human DHP Gene

Specific FISH signals generated from biotinylated genomic DNA of the DHP gene were observed on the long arm of human chromosome 8, in the majority of the metaphase spreads examined (fig. 2). No other reproducible signal was observed on metaphase chromosomes. Thus, FISH assigned the human DHP gene to 8q22.

Mutation Analysis of Genomic DNA

Sequence analysis of exons 1–9 was performed by use of genomic DNA from subjects 1–6, resulting in the

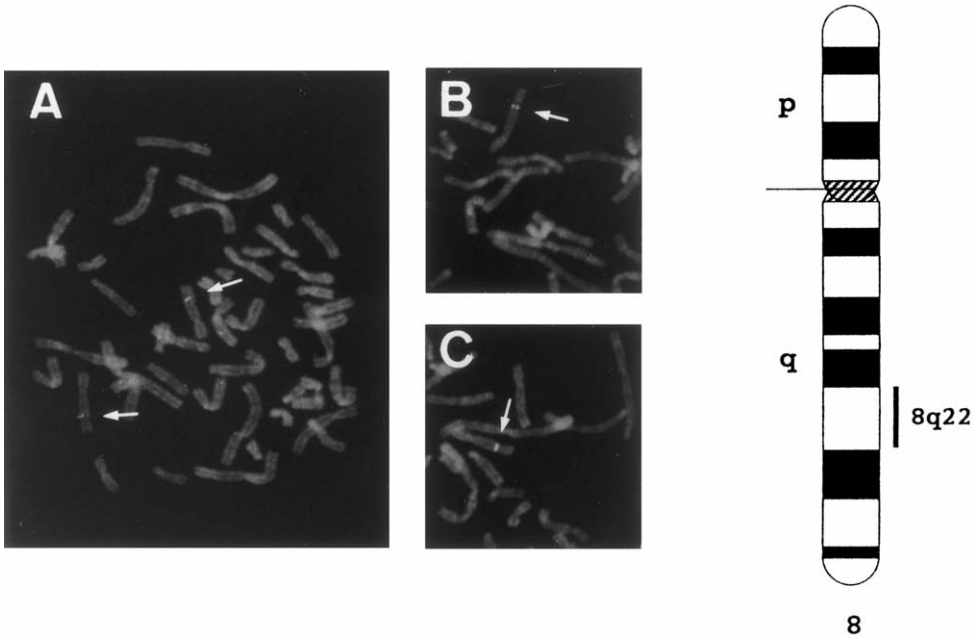

Figure 2 Chromosomal localization of DHP gene on R-banded human chromosomes. Specific FITC signals in the region of chromosome 8q22 are indicated by arrows (*A*–*C*). A schematic presentation of human chromosome 8 and of the location of the DHP gene is shown on the right.

Table 3

Genotype of the Human DHP Gene in DHP-Deficient Patients

Family	Subject	Genotype	Effect[a]
1	1	A1001G/Insertion A[b]	Q334R/Frameshift[b]
2	2	A1001G/A1001G	Q334R/Q334R
	3	A1001G/A1001G	Q334R/Q334R
3	4	A1001G/G1303A[c]	Q334R/G435R[c]
4	5	G203C/C1468G	T68R/R490T
5	6	T1078C/T1078C	W360R/W360R

[a] On DHP protein or mRNA.

[b] Single-base insertion of A at nucleotide position +812 to +814, leading to a frameshift from codon 272 and to a premature termination of translation at codon 287.

[c] Whether Q334R and G435R are actually on the different alleles in this subject could not be definitively determined.

identification of one frameshift mutation and five missense mutations (table 3). Subjects 2 and 3 were shown to be homozygous for an A1001G (Q334R) transition in exon 6, whereas subjects 1 and 4 were shown to be heterozygous for the same mutation. Since the A1001G transition creates a recognition site for *Msp*I, PCR-RFLP analysis to detect this mutation was performed in subjects 1–3 and their family members (fig. 3). The amplification products from subjects 2 and 3 were digested completely by *Msp*I and showed 217- and 145-bp fragments, indicating homozygosity for this mutation. Subject 1 and her mother, two children of subject 2, both parents of both subject 2 and subject 3, and subject 4 and her mother showed the 362-, 217-, and 145-bp fragments, consistent with heterozygosity for this mutation. Subject 1 was also shown to be heterozygous for a single-base insertion at nucleotide positions 812–814 (InsA) in exon 5. This insertion leads to a frameshift at codon 272 and causes premature termination of translation at codon 287. Since the father of subject 1 was found to be a heterozygote for InsA (data not shown), subject 1 was confirmed to be a compound heterozygote for Q334R and InsA. Subject 4 was shown to be heterozygous for a G1303A transition (G435R) in exon 8; however, this mutation was not identified in either her mother or her sibling. Since her father was not available for any biochemical or molecular analysis, and since we did not succeed in a long PCR using genomic DNA from patient 4 to amplify exons 6–8 at a stretch, we could not strictly determine whether Q334R and G435R are actually on the different alleles in subject 4, although it seems likely that subject 4 is indeed a compound heterozygote for both Q334R and G435R. Subject 5 was shown to be a heterozygote for a G203C transition (T68R) in exon 1 and for a G1468C transition (R490T) in exon 9. The mother was shown to be heterozygous for R490T, whereas the father was shown to be heterozygous for T68R. Therefore, subject 5 was confirmed to be a compound heterozygote for both T68R and R490T. Finally, subject 6 was shown to be homozygous for a T1078C transition (W360R) in exon 6. Since this mutation causes no change in restriction-enzyme site, an SSCP analysis of the exon 6 PCR fragment was performed on subject 6 and her family members. SSCP analysis showed that both the (consanguineous) parents and the maternal grandfather were heterozygous for this mutation (fig. 4).

Population Study of Q334R and W360R

In order to establish the frequency of the Q334R and W360R mutation, either PCR-RFLP analysis (in the case of Q334R) or SSCP analysis (in the case of W360R) was performed on 100 unrelated Japanese and 200 Cauca-

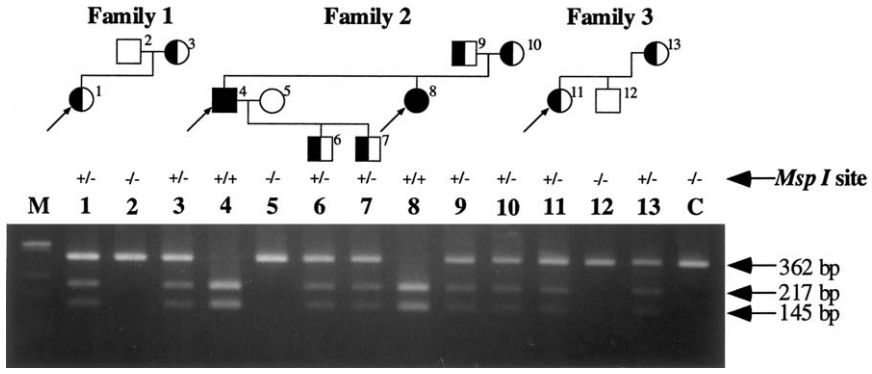

Figure 3 PCR-RFLP analysis to detect Q334R mutant alleles. Partial pedigrees of families 1–3, as well as the results of *Msp*I digestion of PCR products, are shown. The 362-bp PCR products of exon 6 were digested with *Msp*I and were analyzed on a 2% agarose gel. In the presence of the A1001G transition (Q334R), *Msp*I digestion generates two fragments, of 217 and 145 bp. Lane M, Molecular size marker. Lane 1, Subject 1. Lane 2, Mother of subject 1, Lane 3, Father of subject 1. Lane 4, Subject 2. Lane 5, Wife of subject 2. Lane 6, Child 1 of subject 2. Lane 7, Child 2 of subject 2. Lane 8, Subject 3. Lane 9, Mother of subjects 2 and 3. Lane 10, Father of subjects 2 and 3. Lane 11, Subject 4. Lane 12, Sibling of subject 4. Lane 13, Mother of subject 4. Lane C, Normal control. A plus sign (+) denotes that the individual is positive for the *Msp*I site, and a minus sign (−) denotes that the individual is negative for the *Msp*I site.

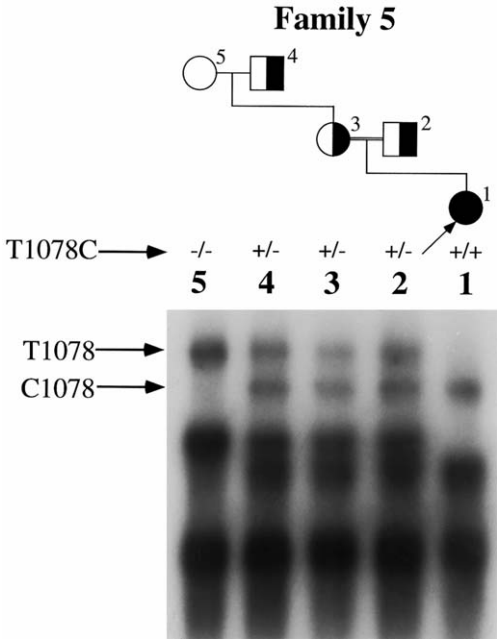

Figure 4 SSCP analysis of exon 6. SSCP analysis of case 6 and her family members was performed as described in the Subjects, Material, and Methods section. Lane 1, Subject 6. Lane 2, Father. Lane 3, Mother. Lane 4, Maternal grandfather. Lane 5, Maternal grandmother. Note that the case is homozygous for C1078, whereas the father, mother, and maternal grandfather are heterozygous. The maternal grandmother has two normal T1078 alleles. Results were confirmed by sequence analysis.

sian control samples, respectively. The PCR-RFLP analysis to detect Q334R revealed no carriers of this mutation (data not shown), suggesting that Q334R is not common in the Japanese population; the SSCP analysis to detect W360R did also not reveal any mutant allele (data not shown), suggesting that W360R is not common in the Caucasian population.

Expression Analysis of Mutated Alleles

In order to establish whether the identified mutations are responsible for the DHP-deficient phenotype, these mutations were introduced into pCR3-hDHP-WT expression plasmid by site-directed mutagenesis and subsequently were expressed transiently in COS-7 cells. Although endogenous DHP activity could be detected in mock-transfected COS-7 cells (mean ± SD = 0.365 ± 0.045 nmol/min/mg protein; $n = 6$), expression of pCR3-hDHP-WT resulted in an ~24-fold induction of DHP activity (8.625 ± 0.187 nmol/min/mg protein; $n = 6$), similar to the activity reported for the rat liver enzyme (Kikugawa et al. 1994). The mean ± SD ($n = 6$) activities in COS-7 cells transfected with the mutant constructs pCR3-hDHP-T68R, pCR3-hDHP-InsA, pCR3-hDHP-Q334R, pCR3-hDHP-W360R, pCR3-hDHP-G435R, and pCR3-hDHP-R490T were 1.493% ± 0.589%, 1.355% ± 0.440%, 2.511% ± 0.963%, 1.155% ± 0.747%, 5.114% ± 0.871%, and 1.711% ± 0.444%, respectively, of the exogenous activity in pCR3-hDHP-WT–transfected COS-7 cells. These results imply that all mutant plasmid–transfected COS-7 cells exhibited a severe reduction of enzymatic activity ($P < .005$). However, the activity observed in pCR3-hDHP-G435R–transfected COS-7 cells was significantly higher than that in the other mutant plasmid–transfected COS-7 cells ($P < .005$). Expression of wild-type and mutant plasmid–transfected COS-7 cells was further investigated, by western and northern blot analysis. Western blot analysis revealed a significant reduction of DHP immunostainable protein in the pCR3-hDHP-InsA–, pCR3-hDHP-W360R–, and pCR3-hDHP-G435R–transfected COS-7 cells, suggesting that the stability or biosynthesis of these mutant proteins is reduced (fig. 5). Other mutant plasmids (pCR3-hDHP-T68R, pCR3-hDHP-Q334R, and pCR3-hDHP-R490T) expressed equal amounts of DHP immunostainable protein, compared with the wild-type construct. By contrast, northern blot analysis showed no significant reduction of DHP mRNA expression for any mutant plasmid–transfected COS-7 cells, indicating that all mutant DHP mRNAs are properly transcribed, processed, and equally stable (fig. 6).

Discussion

In this article we have described the genomic structure of the DHP gene and have shown unequivocally that mutations in this gene provide the molecular basis for

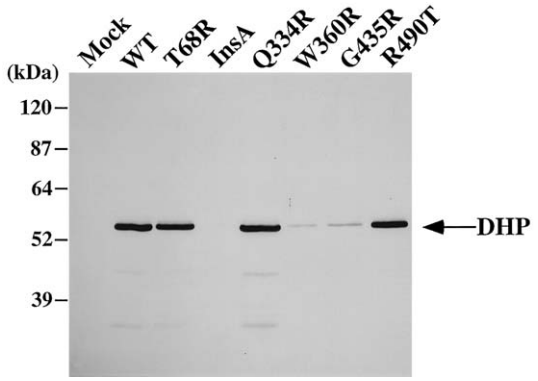

Figure 5 Western blot analysis of cell extracts from COS-7 cells transfected with wild-type and mutant expression plasmids. Each lane contains 10–17 μg cell extracts, quantitatively normalized for β-galactosidase activity and hybridized with polyclonal antibody to rat liver DHP. COS-7 cells were transfected with either pCR3 and pSV-βGal (Mock), pCR3-hDHP-WT and pSV-βGal (WT), pCR3-hDHP-T68R and pSV-βGal (T68R), pCR3-hDHP-InsA and pSV-βGal (InsA), pCR3-hDHP-Q334R and pSV-βGal (Q334R), pCR3-hDHP-W360R and pSV-βGal (W360R), pCR3-hDHP-G435R and pSV-βGal (G435R), or pCR3-hDHP-R490T and pSV-βGal (R490T).

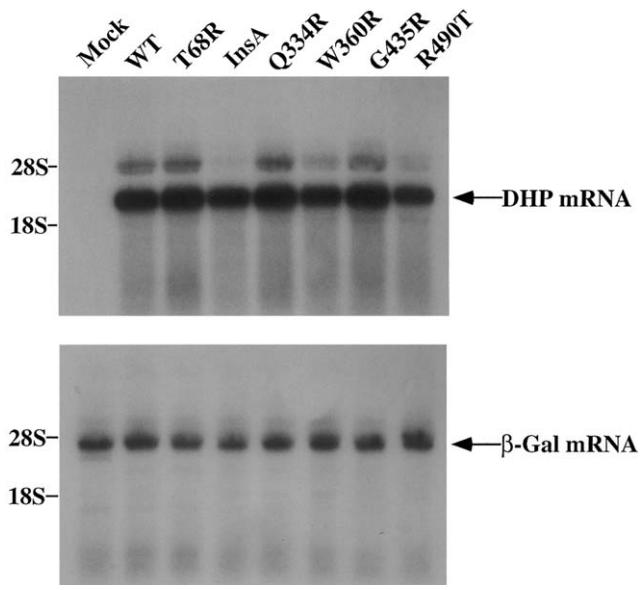

Figure 6 Northern blot analysis of total RNA from COS-7 cells transfected with wild-type and mutant expression plasmids. Each lane contains 5 μg total RNA hybridized with human DHP cDNA (*upper panel*) and *E. coli* β-galactosidase cDNA (*lower panel*). COS-7 cells were transfected with either pCR3 and pSV-βGal (Mock), pCR3-hDHP-WT and pSV-βGal (WT), pCR3-hDHP-T68R and pSV-βGal (T68R), pCR3-hDHP-InsA and pSV-βGal (InsA), pCR3-hDHP-Q334R and pSV-βGal (Q334R), pCR3-hDHP-W360R and pSV-βGal (W360R), pCR3-hDHP-G435R and pSV-βGal (G435R), or pCR3-hDHP-R490T and pSV-βGal (R490T).

human DHP deficiency. Since DHP is expressed only in liver and kidney, enzymatic confirmation of this defect has been performed in only two symptomatic patients. The elucidation of the genomic structure of the DHP gene provides a tool for confirmation of DHP deficiency, without the need to take a liver or kidney biopsy. In addition, molecular analysis of the DHP gene allows unequivocal detection of heterozygotes, which is not possible by means of conventional metabolic analysis.

The human DHP gene has been assigned to 8q22, by FISH (fig. 2). This locus also has been recognized as one of the common fragile sites and is recombinogenic in several human malignancies (Wood et al. 1993). Moreover, there are several disorders associated with abnormalities of this locus, including Cohen syndrome (Tahvanainen et al. 1994), congenital diaphragmatic hernia (Temple et al. 1994), and cleidocranial dysplasia (Brueton et al. 1992). It would be interesting to investigate a possible relationship between these disorders and the DHP gene.

Mutation analysis of genomic DNA in six DHP-deficient individuals revealed the presence of one frameshift mutation (InsA) and five missense mutations (T68R, Q334R, W360R, G435R, and R490T). Expression analysis of these mutations in COS-7 cells revealed that all of them exhibit severely reduced enzymatic activity, indicating that they are DHP deficiency–causing mutations, although the possibility of allelic interactions in the compound-heterozygous genotypes observed in subjects 1, 4, and 5 remained to be examined. Except for subject 4, all subjects were found to be either homozygous or compound heterozygous for one or two of these mutations and were diagnosed as definitely DHP deficient at the molecular genetic level. Although we could not determine whether Q334R and G435R were on different alleles in subject 4, it is likely that this subject is a compound heterozygote. Further evidence is provided by the detailed chemical analysis of pyrimidine metabolites in the urine of this patient and of her available family members (Sumi et al., in press).

Three mutant proteins (InsA, W360R, and G435R) were expressed in COS-7 cells only at low levels, as judged on the basis of western blot analysis. This reduction could not be due to either reduced transcription rates or mRNA instability, since the mutant mRNAs were expressed at levels equal to those in the wild-type and other mutant constructs. However, since the InsA frameshift mutation leads to a premature termination of translation at codon 287, it is likely that the truncated translation product is either unstable or no longer recognized by the antibody. In addition, reduced protein stability also has been observed in other proteins containing nonsense or missense mutations (Zhang et al. 1996; Hovnanian et al. 1997). The mutation W360R is located at the amino-terminal end of the strongly conserved region in DHP and DHP-related proteins (DRPs) (Hamajima et al. 1996), and this region recently has been shown to be essential to in vitro heterotetramer formation of DRPs (Wang and Strittmatter 1997). Moreover, WD (i.e., Trp-Asp) or WE (i.e., Trp-Glu) repeats, which are implicated in protein-protein interactions in GTP-binding protein (Neer et al. 1994), are observed in this region of DHP and DRP sequences. The amino acid residues WE, at codons 360 and 361, and WD, at codons 406 and 407, in the DHP sequence also may be essential to homotetramer formation of this enzyme. Therefore, the W360R substitution may prevent tetramerization, causing instability of the subunit. By contrast, the G435R mutant protein showed significantly higher activity than was seen in other mutant proteins, despite a reduced steady-state protein level. Gly at codon 435 may be essential to protein stability but not important for catalytic activity of the enzyme. To ascertain whether the reduced steady-state level of these mutant proteins is really due to protein instability, pulse-chase experiments are in progress.

The putative zinc-binding sites of DHP are believed to be located either at His67, His69, and His132 (Matsuda et al. 1996) or at His67, His69, His192, and

His248 and Asp326 (Holm and Sander 1997). The T68R substitution is located between His67 and His69 and may result in the inability to bind a zinc ion. The Q334R substitution is located in the vicinity of Asp326 and also may influence the zinc ion–binding ability. In addition, three substitutions (T68R, Q334R, and R490T) are located at completely conserved residues in human and rat DHP and in mammalian or avian DRPs. Substitutions at these residues may alter the secondary or tertiary structure of this enzyme or may influence the ability to bind to substrates, without affecting protein stability.

Only one of the DHP-deficient subjects described here (i.e., subject 6) shows a clinical phenotype, whereas all other subjects are asymptomatic. The observation that deficiencies in enzymes involved in pyrimidine degradation are not always correlated with a clinical phenotype also has been recognized in dihydropyrimidine dehydrogenase (DPD) deficiency. Moreover, it has been shown that, within a single family, identical DPD genotypes do not necessarily lead to identical phenotypes, indicating a lack of correlation between phenotype and genotype (Fernandez-Salguero et al. 1997; Vreken et al. 1997*a*, 1997*b*, 1997*c*). The present study has provided evidence that molecular defects in the DHP gene, resulting in functional DHP deficiency as judged on the basis of the accumulation of pyrimidine degradation products in bodily fluids of these patients, also do not necessarily lead to a clinical phenotype. Decreased synthesis of the neurotransmitter β-alanine may play a role in the neurological symptoms observed in symptomatic DHP- and DPD-deficient patients (Van Gennip et al. 1997*a*), although the presence of a clinical phenotype is unlikely to be solely dependent on β-alanine homeostasis. We cannot exclude the possibility that the phenotypes in the symptomatic DHP- and DPD-deficient patients, rather than being due to these enzyme defects, are only coincidentally associated with the genotypes of these patients. It is tempting, however, to speculate that DHP is a multifunctional protein and serves other functions common to the DRPs. Homozygosity for protein-reducing mutations is not identified in asymptomatic DHP-deficient subjects. The multifunctionality of the DHP protein may be responsible for the phenotype observed in subject 6. Further biochemical and molecular studies are needed in order to elucidate this point.

Similar to the increased toxicity observed in homozygous and heterozygous DPD-deficient individuals (Wei et al. 1996; Van Kuilenburg et al. 1997), homozygosity for DHP deficiency is likely to cause increased 5-FU toxicity, because of both the accumulation of uracil and thymine and the reduced capacity to metabolize these compounds and their analogues (Hayashi et al. 1996). The molecular analysis of DHP deficiency that has been reported here can be applied to cancer patients who will be treated with pyrimidine analogues such as 5-FU.

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Electronic-Database Information

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

Genbank, http://www.ncbi.nlm.nih.gov/Web/Search/index.html (for human DHP gene, exons 1–10)
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for DHP deficiency)

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