Am. J. Hum. Genet. 59:1268–1275, 1996

Severe and Mild Mutations in *cis* for the Methylenetetrahydrofolate Reductase (MTHFR) Gene, and Description of Five Novel Mutations in MTHFR

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Summary

Methylenetetrahydrofolate reductase (MTHFR) catalyzes the synthesis of 5-methyltetrahydrofolate, a methyl donor in the conversion of homocysteine to methionine. Patients with severe MTHFR deficiency have hyperhomocysteinemia, hypomethioninemia, and a range of neurological and vascular findings with a variable age at onset. We have previously described nine mutations in patients with severe MTHFR deficiency. A mild form of MTHFR deficiency, associated with a thermolabile enzyme, has been proposed as a genetic risk factor for cardiovascular disease and for neural tube defects. We have shown that a common missense mutation (an alanine-to-valine substitution) encodes this thermolabile variant. We now report an additional five mutations causing severe MTHFR deficiency and an analysis of genotype (alanine/valine status) and enzyme thermolability in 22 patients with this inborn error of metabolism. Six of these patients have four mutations in the MTHFR gene—two rare mutations causing severe deficiency and two mutations for the common alanine-to-valine mutation that results in thermolability. Even in severe MTHFR deficiency, the thermolabile variant is frequently observed, and there is a strong relationship between the presence of this variant and increased enzyme thermolability.

Introduction

The identification of hyperhomocysteinemia as a risk factor for the development of arteriosclerosis and vascular disease (Boushey et al. 1995) has generated renewed interest in the enzymes and cofactors involved in homocysteine metabolism. One such enzyme, 5,10-methylenetetrahydrofolate reductase (MTHFR), catalyzes the synthesis of the 5-methyltetrahydrofolate cosubstrate used in the remethylation of homocysteine to methionine. Patients with severe MTHFR deficiency are hyperhomocysteinemic and have low enzyme activity (usually 0%–20% of controls), with a wide range of neurological and vascular symptoms (Rosenblatt 1995). These symptoms, which can present in the neonatal or adolescent period, include mental retardation, seizures, peripheral neuropathy, and thromboses. Biochemical studies of fibroblasts from patients with severe MTHFR deficiency have identified a subset of lines with marked enzyme thermolability at 55°C (Rosenblatt and Erbe 1977). These findings, in conjunction with the observed clinical variability, suggested genetic heterogeneity in this disorder.

An analysis of a population of patients with coronary artery disease led to the description of what appeared to be another form of MTHFR deficiency. In that study, 17% of patients carried a variant of MTHFR with 50% of control activity at 37°C and marked thermolability at 46°C. This variant, which was identified in only 5% of a control population, was predicted to be a risk factor for mild hyperhomocysteinemia and arteriosclerosis (Kang et al. 1991*b*).

Our isolation of a cDNA for MTHFR has allowed us to study severe and mild MTHFR deficiency at the molecular level (Goyette et al. 1994). We have identified nine rare mutations in patients with severe MTHFR deficiency (Goyette et al. 1994, 1995) and a common missense mutation, bp 677 C→T (an alanine-to-valine substitution), which has an allele frequency of ~35% in the general population (Frosst et al. 1995; Jacques et al. 1996). The latter mutation results in enzyme thermolability in an in vitro expression system (Frosst et al. 1995). In vivo, the mutation in the heterozygous or homozygous state is associated with reduced specific activity at 37°C and increased thermolability at 46°C in lymphocyte extracts (Frosst et al. 1995; van der Put et al. 1995; Kluijtmans et al. 1996; Christensen et al., in press). Furthermore, individuals homozygous for the

Received May 30, 1996; accepted for publication September 10, 1996.

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0002-9297/96/5906-0013$02.00

mutant allele show significantly increased levels of plasma homocysteine, particularly when their plasma folate is in the low normal range (Frosst et al. 1995; Jacques et al. 1996). This substitution has been proposed as a genetic risk factor for vascular disease. In a recent study of Dutch patients with occlusive arterial disease, the frequency of the homozygous mutant (Val/Val) genotype was ~15% in cases and 5% in controls (Kluijtmans et al. 1996).

The reports of hyperhomocysteinemia in mothers of children with neural tube defects and the recent finding of an increased frequency of the thermolabile variant in Dutch families with spina bifida have led us to propose that the same alanine-to-valine missense mutation may also be a risk factor for neural tube defects (van der Put et al. 1995). A study of Irish families with neural tube defects reached the same conclusion (Whitehead et al. 1995).

In this report, we describe an additional five mutations in severe MTHFR deficiency. We also analyze severely deficient patients for the thermolabile variant and suggest that the thermolability at 55°C originally reported in some of these patients may be due to the presence of the alanine-to-valine polymorphism, in addition to the two mutations that result in severe enzymatic deficiency. Consequently, some of these patients have three or four mutations in the MTHFR gene.

Patients, Material, and Methods

Patients

Patients/cell lines 354, 355, 356, 458, 670, 735, 1084, 1396, 1554, 1627, 1654, 1767, 1772, 1779, 1807, 1834, and 1863 have been described elsewhere (Rosenblatt et al. 1992; Goyette et al. 1994, 1995). Cell lines 1871 and 1872 are from the mother and father, respectively, of brothers 1779 and 1834. Cell line TL22 is from the mother of 1627.

Of the three patients for whom new mutations are described, 1772, a Caucasian male, came to medical attention because of failure to thrive and irregular respiration at 2 wk of age; 670 (family 8, SS) (Erbe 1986) was a Japanese female with developmental delay and seizures who died at age 9 mo; and 1767, the daughter of an African-American father and a Caucasian mother, had lethargy and failure to thrive at age 1 mo and was admitted to hospital at age 3 mo with seizures, apnea, and hypotonia.

Of the remaining patients, 1826 was an Australian female who had an atypical presentation with megaloblastic anemia at age 18 years; 1951 is a Caucasian male who presented in the 1st year of life with developmental delay and seizures and developed gait problems and hyperactivity at age 4 years; 2006 is a Caucasian female who was diagnosed at age 14 years with progressive cognitive and neuropsychiatric problems and developed a progressive gait dysfunction and absence seizures; 2073 is a Caucasian male who developed spastic paraplegia and leukodystrophy; and 2255 is a Saudi Arabian female who had school difficulties at age 12 years, stroke at age 15 years, and developed dementia and spastic paraplegia.

Enzyme Assay

Enzyme activity and thermolability were assessed in crude extracts from cultured fibroblasts obtained from the Repository for Mutant Human Cell Strains, Montreal Children's Hospital. The fibroblasts were lysed by addition of 150 µl of 0.05% Triton X-100 in 100 mM potassium phosphate buffer. Enzyme activity was determined by a modification (Christensen et al., in press) of a previous method (Rosenblatt and Erbe 1977) as described in the following: Cell extracts were incubated for 60 min at 37°C in a reaction mixture containing 180 mM phosphate buffer, 3.5 mM menadione, 1.4 mM EDTA, 7.6 mM ascorbic acid, 70 µM flavin adenine dinucleotide (FAD), and 300 µM [^{14}C]-methyltetrahydrofolate in a total volume of 143 µl. The extract was heated at 55°C for 20 min in the presence of FAD, before the addition of substrate. The reaction was terminated by the addition of 125 µl of 600 mM sodium acetate, pH 4.5. After the addition of 50 µl of 100 mM formaldehyde and 75 µl of 400 mM dimedone, the mixture was boiled for 12 min and subsequently cooled on ice. Two and a half milliliters of toluene were then added to each sample, and the tubes were vortexed twice for 15 s. Formation of the [^{14}C]-radiolabeled formaldehyde-dimedone adduct was quantified by scintillation counting of the supernatant after centrifugation of the samples for 10 min at 1,000 rpm. Protein was determined by the method of Lowry using BSA as standard, and 0.1–0.2 mg protein was used per assay after demonstrating that the enzymatic activity was linear with protein concentration in this range (data not shown). Enzyme activity was expressed as nmol formaldehyde formed per mg protein/h; the mean ± SD for six control lines was 13.3 ± 4.6, with a range of 7.6–20.3. If the residual MTHFR activity was <30% after heating at 55°C for 20 min, the phenotype was designated as "thermolabile."

Mutation Analysis

DNA was isolated from cultured fibroblasts. Identification of unknown mutations was performed by SSCP analysis of individual exons (table 1) and direct sequencing of PCR fragments as described by Goyette et al. (1994). Confirmation of sequence changes was performed by restriction digestion of a new PCR fragment, using naturally occurring or artificially created restric-

Table 1

Primers for PCR Amplification of Exons in Mutation Analysis of MTHFR

Exon[a] and Primer Type	Primers (5′→3′)	Location[b]	Fragment Size[c] (bp)
1:			
Sense	AGCCTCAACCCCTGCTTGGAGG	c	271
Antisense	TGACAGTTTGCTCCCCAGGCAC	i	
2:			
Sense	GGAAGGCAGTGACGGATGGTAT	i	373
Antisense	ACCAAGTTCAGGCTACCAAGTGG	i	
5:			
Sense	CACTGTGGTTGGCATGGATGATG	i	392
Antisense	GGCTGCTCTTGGACCCTCCTC	i	
6:			
Sense	TGCTTCCGGCTCCCTCTAGCC	i	251
Antisense	CCTCCCGCTCCCAAGAACAAAG	i	

[a] Exon designation is based on the translation initiation codon of the smaller MTHFR isoform, 70 kD. (Frosst et al. 1995).

[b] c = cDNA; i = intronic.

[c] Fragment size is the size of the exon plus flanking intronic sequences.

tion sites (ACRS), or by allele-specific oligonucleotide (ASO) hybridization (Goyette et al. 1995). The presence of the alanine-to-valine missense mutation was determined by restriction digestion with *Hin*fI as reported by Frosst et al. (1995).

Results

Identification of Mutations in Patients with Severe MTHFR Deficiency

Patient 1772 carried a G→C substitution at bp 164, in exon 1 (table 2*A*), which converted an Arg residue to a Pro residue; the change creates a *Hae*III restriction site. Restriction digestion with *Hae*III was used to confirm the mutation in patient 1772 (fig. 1*A*) and to screen 54 independent control chromosomes. All controls were negative for this substitution. The second mutation in patient 1772 was a 3′ splice-site mutation, a G→T substitution in the splice-acceptor dinucleotide (AG) in intron 1 preceding bp 249 (bp 249−1). The change creates an *Afl*III restriction site, which was used to confirm the change in this patient (fig. 1*B*).

Patient 670 is homozygous for a GC→TT substitution at bp 458–459, in exon 2 (table 2*A*), which changes a Gly residue to a Val residue. The Gly residue is evolutionarily conserved in the bacterial enzyme (Goyette et al. 1994). ASO hybridization was used to confirm this double change in the patient and to screen 54 independent chromosomes, which proved to be negative (figs. 2*A* and 2*B*). The G→T substitution at bp 458 is responsible for the amino acid change, while the C→T substitution at bp 459 is a silent change. Both substitutions were tested independently by ASO hybridization; neither of the individual substitutions nor the double change was seen in 54 control chromosomes.

Patient 1767 was a compound heterozygote for a T→C substitution at bp 980, in exon 5 (table 2*C*), which converted an evolutionarily conserved Leu residue (Goyette et al. 1994) to a Pro residue, and for a C→T substitution at bp 1141, in exon 6, which changed an Arg residue to a Cys residue. The former substitution was confirmed by restriction digestion for a *Taq*I restriction site that was artificially created for the control fragment; this substitution was not seen on 54 control chromosomes (fig. 1*C*). The second mutation was confirmed by ASO hybridization (figs. 2*C* and 2*D*) and was not seen on 74 control chromosomes.

SSCP analysis and sequencing also identified a polymorphism at bp 1178+31 in intron 6. The T nucleotide was identified in 71% of alleles ($n = 84$) in a sample of Caucasian DNAs, while the C nucleotide was seen in 29% of alleles ($n = 84$).

Correlation of Ala/Val Genotype with Thermolability in Patients with Severe MTHFR Deficiency

Twenty fibroblast lines from unrelated patients with severe MTHFR deficiency, fibroblasts from two affected sibs (tables 2*A*, 2*B*, and 2*C*), and three obligate heterozygous parental cell lines (table 2*D*) were tested for the Ala-to-Val mutation. Tables 2*A* and 2*B* summarize the results for patients who are homozygous for the Ala or Val allele, respectively. Table 2*C* shows the data for patients who are heterozygous for the thermolabile variant. The mutations accounting for the severe deficiency and the level of spe-

Table 2

Analysis of Thermolabile Polymorphism (A→V) in Patients and Obligate Heterozygotes for Severe MTHFR Deficiency

Patient or Obligate Heterozygote No.	Mutation (bp change)	Mutation (amino acid change)	Specific Activity (% of control)	A/V Status	Thermolability
	A. Patients Homozygous for Ala Allele				
354/355[a]	792+1G→A/?	N.A./?	19/14[b]	A/A	No
670	G458T/G458T	G→V/G→V	4	A/A	Yes
735	C692T/C692T	T→M/T→M	2	A/A	ND
1084	C692T/C692T	T→M/T→M	0	A/A	ND
1627	C559T/C559T	R→X/R→X	1	A/A	ND
1654	?/?	?/?	0	A/A	ND
1772	249-1G→T/G164C	N.A./R→P	1.6	A/A	No
1779/1834[a]	G482A/?	R→Q/?	6/7[b]	A/A	Yes
2255	?/?	?/?	30	A/A	Yes
	B. Patients Homozygous for Val Allele				
356	C985T/C985T	R→C/R→C	20	V/V	Yes
458	G167A/C1015T	R→Q/R→C	10	V/V	Yes
1554	C559T/C559T	R→X/R→X	0	V/V	ND
1807	C764T/C764T	P→L/P→L	3	V/V	Yes
1826	?/?	?/?	28	V/V	Yes
1951	?/?	?/?	7	V/V	Yes
	C. Patients Heterozygous for Thermolabile Variant				
1396	G167A/C1081T	R→Q/R→C	14	A/V	Yes
1767	T980C/C1141T	L→P/R→C	2	A/V	No
1863	G482A/?	R→Q/?	14	A/V	Yes
2006	?/?	?/?	7	A/V	Yes
2073	?/?	?/?	33	A/V	Yes
	D. Obligate Heterozygotes				
1871	G482A/N	R→Q/N	44	A/A	No
1872	?/N	?/N	39	A/V	Yes
TL22	C559T/N	R→X/N	17	A/V	Yes

NOTE.—Thermolability is defined as <30% residual activity after heating at 55°C for 20 min. Mutations reported in this paper are underlined. All other mutations were described by Goyette et al. (1994, 1995). ? = unidentified mutation; NA = not applicable; ND = not determined, since specific activity was too low for accurate measurement of heat-resistant activity; N = normal allele in obligate heterozygotes.

[a] The two patients are sibs.

[b] Specific activity for each of the sibs.

cific activity (% of control) are listed in the table, where available. In some patients, the level of specific activity was too low to determine accurately the residual activity after heating. All patients homozygous for the mutant valine allele (table 2*B*) had thermolabile enzyme; in one patient (1554), the residual activity after heating could not be measured, since the specific activity before heating was too low. Three patients without the valine mutation (table 2*A*) had thermolabile enzyme, suggesting the presence of other MTHFR mutations that might affect protein stability. Of the five patients who were heterozygous for the thermolabile variant (table 2*C*), four cell lines had thermolabile enzyme, while one line had thermostable enzyme. Table 2*D* shows the results for obligate heterozygotes. 1871 and 1872 are the mother and father, respectively, of two brothers—patients 1779 and 1834 in table 2*A*. TL22 is the mother of patient 1627 in table 2*A;* this family was first described by Kang et al. (1991*a*). The two individuals in table 2*D* who have thermolabile activity (1872 and TL22) carry one copy of the valine allele, in addition to the one copy of a "severe" mutation.

Discussion

Our ongoing analysis of severe MTHFR deficiency has resulted in the identification of a total of 14 muta-

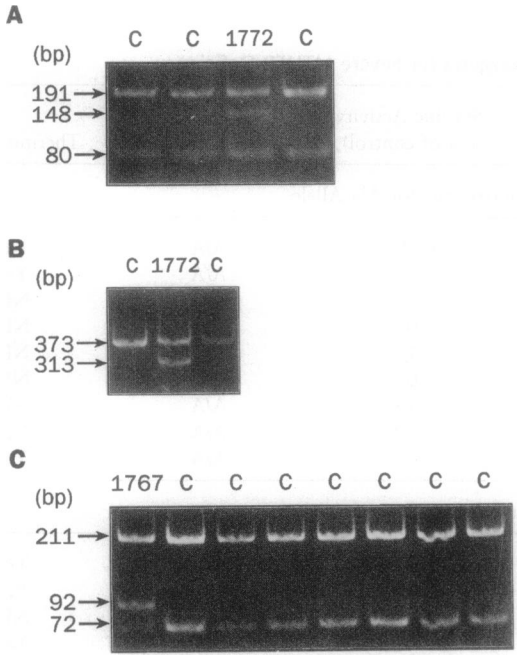

Figure 1 Restriction endonuclease analysis of three mutations (for primers, see table 1). *A*, *Hae*III restriction analysis of exon 1 PCR products from patient 1772 and three controls (C). Expected fragments: control allele—191 and 80 bp; mutant allele—148, 80, and 43 bp. The 43-bp fragment is not shown in the figure. *B*, *Afl*III restriction analysis of exon two PCR products from patient 1772 and two controls (C). Expected fragments: control allele—373 bp; mutant allele—313 and 60 bp. The 60-bp fragment is not shown in the figure. *C*, *Taq*I ACRS analysis of a PCR fragment of exon 5 (303 bp), amplified from the 5′ sense primer of exon 5 in table 1, and an antisense primer, creating a *Taq*I restriction site in the control allele (5′-GTGG-TAGCCATCTCGCGGTCG-3′). The *Taq*I restriction analysis shows patient 1767 and seven controls (C). Expected fragments: control allele—211, 72, and 20 bp; mutant allele—211 and 92 bp. The 20-bp fragment is not shown in the figure.

tions in 14 families (Goyette et al. 1994, 1995; present study). In this report, we described five novel mutations to complete the mutational analysis in three patients. Combined with our earlier work, both mutations have been identified in 11 patients with severe MTHFR deficiency. The high degree of genetic heterogeneity is quite striking; each severe mutation has been present in only one or two families.

The five novel mutations reported here are not likely to be benign polymorphisms. The mutation in the 3′ splice site (249-1 G→T) occurs in the critical AG dinucleotide in the intronic consensus sequence and is likely to affect normal splicing of the mRNA; mRNA for this patient has not yet been examined. The other four mutations are missense substitutions, which were not present in a large panel of control DNAs. Two of these—the Gly-to-Val and Leu-to-Pro substitutions (G458T and T980C, respectively)—affect amino acid residues that are evolutionarily conserved in bacteria (Goyette et al. 1994) and are therefore likely to be critical to enzyme function. The Arg-to-Pro and Arg-to-Cys substitutions (G164C and C1141T, respectively) convert a charged residue to an uncharged residue and for that reason are likely to be deleterious.

Purified porcine MTHFR is a homodimer of 77-kD subunits. The polypeptide can be proteolytically cleaved into a N-terminal 40-kD catalytic domain and a C-terminal 37-kD catalytic domain that contains the binding site for the allosteric inhibitor, S-adenosylmethionine (Matthews et al. 1984; Sumner et al. 1986). The Arg-to-Cys substitution (C1141T) is the only mutation identified thus far in the putative C-terminal regulatory domain. It is important to note, however, that the first identified cDNA of 1.3-kb encoded N-terminal sequences primarily, and therefore the first mutations in MTHFR were identified in this region of the protein. Our recent isolation of the remainder of the cDNA encoding the C-terminal region will facilitate the identification of mutations in this domain (Frosst et al. 1995).

The three patients harboring the five new mutations were characterized by neonatal onset of symptoms with an extremely severe course, including death at 9 mo for patient 670 (Erbe 1986; Rosenblatt et al. 1992). All three patients had <5% of control MTHFR activity, indicating that the mutations are particularly deleterious. Overall, there is reasonable correlation between genotype and residual enzyme activity in severe MTHFR deficiency, although we have described one pair of sibs with MTHFR deficiency and dramatic differences in phenotype (Goyette et al. 1995). In the 11 patients with severe MTHFR deficiency in whom both mutations have been identified, 8 had onset of symptoms in the 1st year of life, while 3 had adolescent onset of symptoms with a milder course.

The identification of the common thermolabile MTHFR mutation in families with severe deficiency adds another level of complexity to the analysis of genotype and phenotype. The presence of the thermolabile mutation in these families is likely a function of its high frequency in the general population overall (~35% of alleles). Since its frequency in 20 patients with severe MTHFR deficiency (17/40 alleles, or 42%) is not very different from that found in the general population, it is likely that the thermolabile polymorphism is the older of the two types of mutations. Consistent with this hypothesis is the finding of the thermolabile polymorphism in Caucasian and non-Caucasian populations, albeit at different frequencies (Motulsky 1996).

Thermolability for MTHFR was first reported in severe MTHFR deficiency as residual activity after heating at 55°C (Rosenblatt and Erbe 1977). Approximately 10 years later, thermolability of MTHFR was assayed at

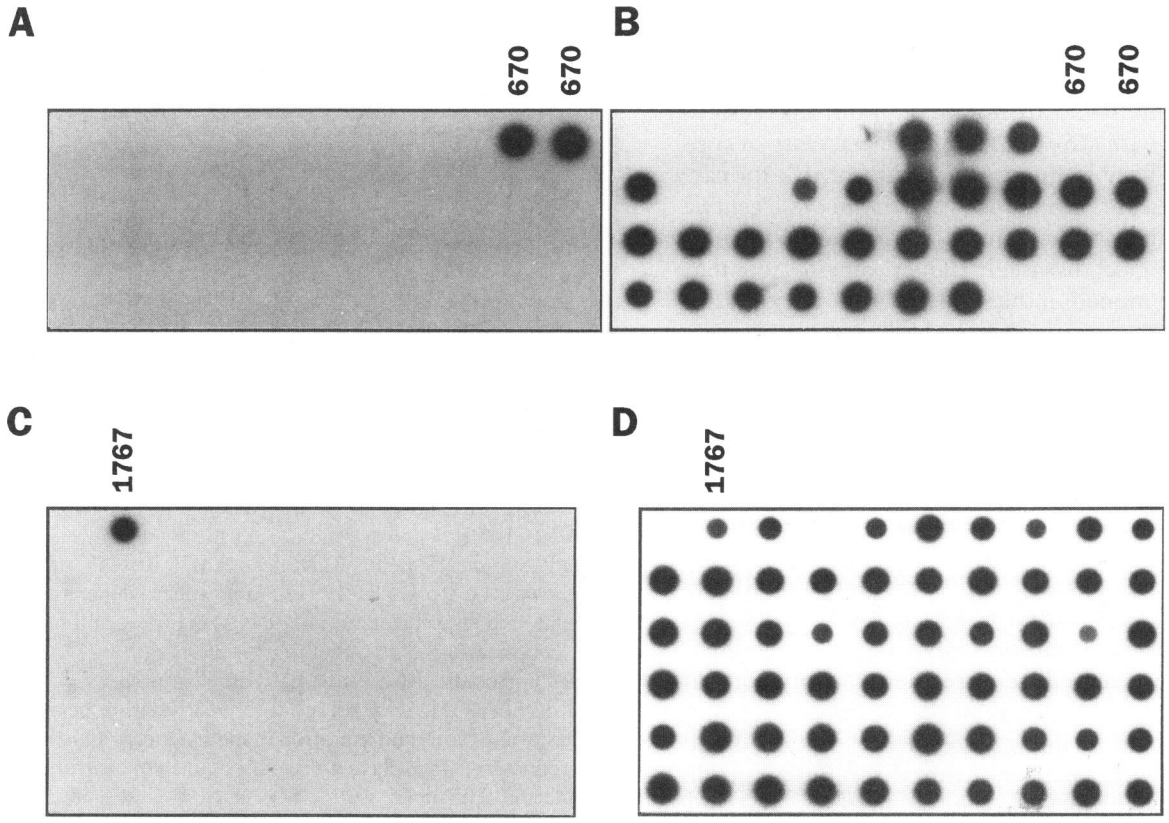

Figure 2 ASO hybridization analysis of two mutations (for primers, see table 1). *A*, Hybridization of mutant oligonucleotide (bp 458/459 = TT) to exon 2 PCR products from patient 670 and 28 controls. Only the DNA from two independent samples of patient 670 hybridized to this probe. *B*, Hybridization of normal oligonucleotide (bp 458/459 = GC) to stripped dot blot from panel *A*. All control DNAs hybridized to this probe. *C*, Hybridization of mutant oligonucleotide (1141T) to exon 6 PCR products from patient 1767, other patients with MTHFR deficiency, and 37 controls. Only the DNA from patient 1767 hybridized to this probe. *D*, Hybridization of normal oligonucleotide (1141C) to stripped dot blot from panel *C*. DNA from 1767 and all others hybridized to this probe.

46°C and proposed to be a risk factor for coronary artery disease (Kang et al. 1991*b*). Our data suggest that the alanine-to-valine missense mutation is likely the cause of thermolability in both studies at both temperatures. However, there are clearly other mutations in severe MTHFR deficiency that can confer thermolability, since three of the patients who were homozygous for the alanine allele had altered thermolability. On the other hand, it is unlikely that there are other common thermolabile alleles in the general population, since very good correlation has been observed between the alanine/valine genotype and thermolability in several different studies of cases and controls in cardiovascular disease and in neural tube defects (van der Put et al. 1994; Kluijtmans et al. 1996; Christensen et al., in press).

Cell line TL22 is the mother of patient 1627, who was previously shown to be homozygous for a nonsense mutation (Goyette et al. 1994). In the original description of this family, the mother had been predicted to be a genetic compound for a severe MTHFR mutation and for thermolabile reductase; her enzyme activity had been shown to be 17% of control values (Kang et al. 1991*a*). We have now shown that this woman does in fact carry two mutations: the nonsense mutation that she passed on to her son, patient 1627 (table 2*A*), and the alanine-to-valine missense mutation that was not inherited by the patient (table 2*D*). These data indicate that the woman's two mutations are on separate alleles.

The other two obligate heterozygotes in our study, cell lines 1872 and 1871, are the father and mother, respectively, of two previously reported probands with one mutation identified thus far (Goyette et al. 1994). The two brothers (patients 1779 and 1834) do not carry the alanine-to-valine mutation; their identified "severe" mutation was inherited from their mother (1871), who is negative for the thermolabile variant. Since the father (1872) is a carrier of a valine allele that was not passed on to his sons, the father must have a severe MTHFR mutation (as yet unidentified) on one allele and the thermolabile variant on the second allele.

The obligate heterozygotes described may have <50% of the specific activity of controls, since they are genetic compounds for the severe and thermolabile alleles. Individuals who are homozygous for the thermolabile variant, with <50% of the specific activity of controls at 37°C, have been proposed to be at risk for cardiovascular disease. The reduced activity in these genetic compounds might place them at risk for cardiovascular and/or neurological disease. It is interesting to note that the above-mentioned father (1872) had unexplained paraparesis at the age of 65 years. However, since severe MTHFR deficiency is a rare disorder, it will be difficult to document the phenotype of these types of compounds in a large series of obligate heterozygotes.

The probands with early onset of symptoms in severe MTHFR deficiency are already at risk for a life-threatening disorder, by virtue of their two severe mutations. The additional one or two copies of the thermolabile mutation in some of the patients may not dramatically alter their clinical course. However, the patients with adolescent onset of symptoms have higher levels of enzyme activity and a milder course; their phenotype could be influenced by the presence of the thermolabile mutation, which could further reduce their activity. To add to the complexity, patients with three mutations in MTHFR (two distinct "severe" mutations and one "thermolabile" mutation) may differ in phenotype, depending on the phase of these mutations. It is possible that patient 1767 (table 2C) does not have demonstrable thermolabile enzyme because the valine mutation is in *cis* with a "severe" mutation with virtually no activity. In addition to studying the clinical consequences of severe and mild mutations in *cis*, it will be of interest to determine whether and how the severe and thermolabile mutations on the same subunit (or even on different subunits) interact biochemically with respect to enzyme activity and stability.

Acknowledgments

We thank Carolyn Mandel for preparation of the manuscript and the many physicians and families who have contributed to the analysis of patients with MTHFR deficiency. P.G. is supported by a studentship award from the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche. B.C. is a Renouf Fellow of the Royal Victoria Hospital Research Institute. D.S.R. and R.R. are Principal Investigators of the Medical Research Council Group in Medical Genetics.

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