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RESEARCH ARTICLE

cblE Type of Homocystinuria Due To Methionine Synthase Reductase Deficiency: Functional Correction by Minigene Expression

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The *cblE* type of homocystinuria is a rare autosomal recessive disorder caused by impaired reductive activation of methionine synthase. Although earlier biochemical studies proposed that the methionine synthase enzyme might be activated by two different reducing systems, mutations were reported in only the methionine synthase reductase gene (*MTRR*) in *cblE* patients. The pathogenicity of *MTRR* mutations, however, has not yet been tested functionally. We report on nine patients of European origin affected by the *cblE* type of homocystinuria. They presented between 2 weeks and 3 years of age (median age 4 weeks) with anemia, which was macrocytic in only three patients, and with neurological involvement in all but two cases. Bone marrow examination performed in seven patients showed megaloblastic changes in all but one of them. All patients exhibited moderate to severe hyperhomocysteinemia (median plasma total homocysteine [Hcy] 92 μmol/L, range 44–169), while clearly reduced methionine was observed only in four cases. Pathogenic mutations were identified in both parental alleles of the *MTRR* gene in all patients. Five known (c.903+469T>C, c.1361C>T, c.1459G>A, c.1557-4_1557+3del7, and c.1622_1623dupTA) and three novel mutations (c.7A>T, c.1573C>T, and c.1953-6_1953-2del5) were detected. Importantly, transfection of fibroblasts of *cblE* patients with a wild-type *MTRR* minigene expression construct resulted in a significant approximately four-fold increase of methionine synthesis, indicating correction of the enzyme defect. Our study shows a link between a milder predominantly hematological presentation and homozygosity for the c.1361C>T mutation, but no other obvious genotype-phenotype correlation. The identification of mutations in the *MTRR* gene, together with restoration of methionine synthesis following *MTRR* minigene expression in *cblE* cells confirms that this disease is caused by defects in the *MTRR* gene. Hum Mutat 25:239–247, 2005. **© 2005 Wiley-Liss, Inc.**

KEY WORDS: methionine synthase reductase, MTRR; *cblE*; homocystinuria; megaloblastic anemia; minigene expression; haplotype; SNP

DATABASES:

MTRR – OMIM: 602568, 236270 (*cblE*); GenBank: AF025794.1 (mRNA), NT_006576 (gDNA)

INTRODUCTION

Methionine synthase (EC 2.1.1.13) is a methylcobalamin-dependent cytosolic enzyme required for the remethylation of homocysteine to methionine. Patients with inherited functional deficiency of methionine synthase have been shown to belong to two different complementation groups, *cblG* (MIM# 250940) and *cblE* (MIM# 236270) [Watkins and Rosenblatt, 1988]. Both disorders are rare and inherited in an autosomal recessive manner.

The *cblE* defect mainly manifests in early childhood with failure to thrive, megaloblastic anemia, developmental delay, and cerebral atrophy with white matter abnormalities. Patients exhibit homocystinuria, hyperhomocysteinemia, and often hypomethioninemia

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[Rosenblatt and Fenton, 2001]. Until now, only 17 patients with this disorder have been described worldwide and the spectrum of clinical manifestation remains unclear [Fowler et al., 1997a; Rosenblatt and Fenton, 2001; Zavadakova et al., 2002; Vilaseca et al., 2003].

cblE patient fibroblasts show deficient methionine synthase activity only under limiting reducing agent concentrations, whereas the activity is decreased under all conditions in cells of *cbl*G patients [Rosenblatt et al., 1984; Gulati et al., 1997]. Accordingly, the primary defect in *cbl*G patients is in the methionine synthase molecule itself, whereas *cblE* patients show functional deficiency of methionine synthase due to a defect in a reducing system required to activate this enzyme. The gene for methionine synthase (*MTR*; MIM# 156570) was identified in 1997 and shown to carry pathogenic mutations in *cbl*G patients [Leclerc et al., 1996; Li et al., 1996; Chen et al., 1997].

Leclerc et al. [1998] identified and cloned cDNA corresponding to human methionine synthase reductase (*MTRR*, MIM# 602568) based on homology with sequences of bacterial and *C. elegans* enzymes, and also identified mutations in this gene in *cblE* patients [Leclerc et al., 1998]. Based on this, they concluded that a single protein, MTRR (EC 1.16.1.8), is responsible for the *cblE* defect. Subsequently, Olteanu and Banerjee [2001] expressed *MTRR* cDNA in *E. coli* and showed that it is solely sufficient for the reductive activation of methionine synthase. MTRR is a member of a group of ferredoxin–nicotinamide adenine dinucleotide phosphate ($NADP^+$) reductases containing FMN, FAD and nicotinamide adenine dinucleotide phosphate (reduced form, or NADPH) binding sites. The gene was mapped to human chromosome 5p15.3–p15.2 [Leclerc et al., 1999], is 34-kb long, comprises 15 exons, and is thought to produce cytosolic and mitochondrial mRNA isoforms. So far, 15 pathogenic mutations in the *MTRR* gene have been reported in 13 *cblE* patients [Leclerc et al., 1998; Wilson et al., 1999a; Zavadakova et al., 2002; Vilaseca et al., 2003]. Additionally, two common polymorphisms c.66A>G and c.524C>T have been described [Wilson et al., 1999b; Kahleova et al., 2002].

The question of whether MTRR is the only physiologically important system for the activation of methionine synthase was raised by the description of a possible alternative reducing system by R. Banerjee's group in the same year in which MTRR was identified. They demonstrated that methionine synthase can be activated in a NADPH-dependent reaction requiring a minimum of two redox proteins [Gulati et al., 1997]. These proteins were subsequently purified and identified as soluble cytochrome b_5 (CYB5; MIM# 250790) and the novel reductase 1 (NR1; MIM# 606073) [Chen and Banerjee, 1998; Olteanu and Banerjee, 2003]. However, based on stoichiometry data, they later suggested that under physiological conditions MTRR represents a major pathway in reductive activation of methionine synthase, while the CYB5/NR1 system plays at most a minor role.

In this study, the nature of mutations in the *MTRR* gene in nine homocystinuric patients of European origin, shown to belong to the *cblE* complementation group, were investigated, and correction studies using a wild-type *MTRR* minigene construct and patient fibroblasts were performed to provide conclusive functional evidence that the *cblE* defect is caused by mutations in the *MTRR* gene.

MATERIALS AND METHODS

Patients and Controls

This study includes nine European patients with the *cblE* type of homocystinuria, as summarized in Supplementary Table S1 (available online at http://www.interscience.wiley.com/jpages/1059-7794/suppmat) and Table 1, and one control patient (Patient AX) with isolated functional methionine synthase deficiency but not belonging to the *cblE* or *cbl*G complementation group [Fowler et al., 1997b]. Patients 7 and 2 (Cases 1 and 2 in Zavadakova et al. [2002], respectively) and Patients 4 and 5 (Cases 1 and 2 in Vilaseca et al. [2003], respectively) have been reported earlier. Control blood samples for DNA analysis were obtained as published in Janosikova et al. [2003].

TABLE 1. ***MTRR* Genotypes and Laboratory Findings in cblE Patients**

	Genotype[a]		Origin	Enzyme activity (% of mean control value)[b]	Laboratory findings: At diagnosis		Laboratory findings: At clinical manifestation	
Patient	Paternal allele	Maternal allele			Plasma tHcy (μmol/L)[c]	Plasma/serum-Met (μmol/L)[d]	Hb (g/L)[e]	MCV (fl)[f]
1	c.7A>T	c.7A>T	Turkish	1.9	155	5*	85	85
2	c.903+469T>C	c.903+469T>C	Czech	9.2	92	16	76	96
3	c.903+469T>C	c.903+469T>C	German	8.4	92	22	87	118
4	c.1361C>T	c.1361C>T	Spanish	3.1	91	27	76	103**
5	c.1361C>T	c.1361C>T	Portuguese	6.5	44	18	61	121**
6	c.1573C>T	c.1361C>T	German/ Portuguese	1.5	169	12	128	118**
7	c.1459G>A	c.1622_1623dupTA	Czech	1.5	116	9*	60	90
8	c.903+469T>C	c.1557-4_1557+3del7	Polish	2.3	86	12*	68	104
9	c.903+469T>C	c.1952-6_-2del5	German	5.7	117	17	67	98

[a]+1 corresponds to the A of the ATG translation initiation codon in the reference sequence of the putative cytosolic isoform of the *MTRR* gene; GenBank Accession No. AF025794.1 (mRNA), NT_006576 (gDNA).
[b]Enzyme activity was assayed indirectly by measuring the formation of ^{14}C-methionine from ^{14}C-formate in cultured nontransformed fibroblast (control values, mean ± SD: 2.61 ± 0.75 nmol/16 hr/mg protein, range: 1.42–4.34, n = 16).
[c]Reference range for plasma total homocysteine: 5–20 μmol/L.
[d]Reference range for plasma or serum methionine: 15–40 μmol/L; patients with hypomethioninemia are marked with*.
[e]Reference range for hemoglobin in different age groups. 125–205 g/L (2–4 weeks), 100–180 g/L (1–2 months), 90–140 g/L (2–3 months), 95–135 g/L (3–6 months), 105–135 g/L (6–24 months), 115–135 g/L (2–6 years), 115–155 g/L (6–12 years), 130 g/L (12–18 years).
[f]Reference range for MCV in different age groups: 86–124–fl (2–4 weeks), 85–123 fl (1–2 months), 77–115 fl (2–3 months), 74–108 fl (3–6 months), 70–86 fl (6–24 months), 75–87 fl (2–6 years), 77–95 fl (6–12 years), 78–98 fl (12–18 years); patients with macrocytosis are marked with**.

Metabolite Measurements

Total plasma homocysteine (tHcy) was determined by ion exchange chromatography following dithiothreitol reduction [Brattstrom et al., 1988] or by HPLC using the fluorescence method [Araki and Sako, 1987]. Plasma methionine levels were measured by an amino acid analyzer.

Cell Cultures and Enzyme Assays

Fibroblast cultures were grown from skin biopsies, and routinely maintained in Earle's minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS), 2 mmol/L L-glutamine and antibiotic-antimycotic mixture (Gibco BRL, www.invitrogen.com). Cells were incubated at 37°C in an atmosphere of 50 mL/L CO_2/950 mL/L air, with a relative humidity of 98%.

The activity of the methionine synthase/methionine synthase reductase complex was assayed indirectly by measuring the formation of ^{14}C-methionine from ^{14}C-formate in intact fibroblasts, as previously described [Fowler et al., 1997c]. Somatic complementation analysis was performed by measuring the formation of methionine in patients' cells after fusion with fibroblasts known to belong to the *cblG* and the *cblE* complementation groups, as previously described [Zavadakova et al., 2002]. β-Galactosidase activity was assayed in cell lysates, prepared in potassium phosphate buffer (10 mmol/L, pH 7.4) by repeated freezing and thawing, using the β-Gal Assay Kit (Invitrogen, www.invitrogen.com) with the artificial substrate O-nitrophenyl-β-D-galactopyranoside according to the manufacturer's instructions.

Mutation and Haplotype Analysis

Genomic DNA was isolated from venous blood or cultured fibroblasts after proteinase K treatment by phenol-chloroform extraction [Ausubel et al., 1992] or using the QIAamp DNA Blood Mini Kit (Qiagen, www.qiagen.com). Total RNA was prepared using the guanidinium thiocyanate method [Chomczynski and Sacchi, 1987] and used for preparation of cDNA by reverse transcription with SuperScript II and Oligo(dt)12-18 Primer (Invitrogen).

The entire *MTRR* coding sequence (GenBank Accession No. AF025794.1), with its flanking 5′ and 3′ untranslated regions was amplified in five overlapping segments employing cDNA [Zavadakova et al., 2002]. PCR products were purified and subsequently used for direct sequencing using universal Cy5-labeled primers and the dideoxy chain-termination method, combined with cycle sequencing as described previously [Zavadakova et al., 2002]. In mutation numbering based on cDNA sequence, +1 corresponds to the A of the ATG translation initiation codon in the reference sequence of the putative cytosolic isoform of the *MTRR* gene.

The presence of each nucleotide change observed in cDNA was verified both by sequencing of PCR products and by the PCR-RFLP method, using genomic DNA as the template (GenBank Accession No. NT_023089.13). PCR primers were for exon 2: 5′TCTTAAAGATTGAGGGAGAAT3′ and 5′TATTAAAAAAATGCTGGTGAT3′; for intron 14: 5′GAAGAACTACGGTGGTACA3′ and 5′GCGTTGTGCAGGTTCGTG3′; PCR primers for exons 10, 11, and 12, and for intron 6 were published previously [Zavadakova et al., 2002; Vilaseca et al., 2003]. If a mutation did not create or abolish a restriction site we used artificially generated restriction sites as shown in Table 2. Digestions were carried out overnight with 200–500 ng of the

TABLE 2. Overview of *MTRR* Mutations and Polymorphisms and Their Consequences

	Analysis of DNA mutations						
No. of alleles	Genomic DNA[a]	cDNA[a]	CpG dinucleotide[b]	Restriction site[c]	Observed transcript change	Deduced amino acid change (codon, if noncoding SNP)	Reference
Pathogenic mutations							
6	g.12952T>C	c.903+469T>C	–	HpaII+	r.903_904ins140	p.N30fsX362	Wilson et al. [1999a]
5	g.20611C>T	c.1361C>T	+	XmnI–,*		p.S454L	Vilaseca et al. [2003]
2	g.7A>T	c.7A>T	–	BseRI–		p.R3W	Novel
1	g.22021G>A	c.1459G>A	–	MboII+		p.G487R	Wilson et al. [1999a]
1	g.22116_22122del7	c.15574_1557+3del7	–	SauI–	r. 1462_1557del96	p.V488_K519del	Wilson et al. [1999a]
1	g.24955C>T	c.1573C>T	+	TaqI–		p.R525X	Novel
1	g.25004_25005dupTA	c.1622_1623dupTA	–	MslI–		p.M542fsX542	Zavadakova et al. [2002]
1	g.29114_29118del5	g.1953-6_1953-2del5	–	XbaI-	Unstable allele	unknown	Novel
Polymorphisms[d]							
	g.66A>G	c.66A>G	–	NdeI–		p.I22M	Wilson et al [1999b]
	g.7272C>T	c.524C>T	+	XhoI–		p.S175L	Kahleova et al. [2002]
	g.15052A>G	c.1049A>G	–	BseGI +,*		p.K350R	Novel
	g.20599C>G	c.1349>G	+	HgaI+		p.P450R	Novel
	g.26412G>A	c.1911G>A	+	BcgI–, *		(p.A637)	Novel

[a]+1 corresponds to the A of the ATG translation initiation codon in the reference sequence of the putative cytosolic isoform of the *MTRR* gene; GenBank Accession No. AF025794.1 (mRNA), NT_203089.13 (gDNA).
[b]+, mutation occurs in a CpG dinucleotide; –, mutation does not occur in a CpG dinucleotide.
[c]+, mutation forms a new restriction site; –, mutation abolishes a restriction site; *, an artifically formed restriction site.
[d]The prevalence of variant alleles c.66A>G, c.524C>T, c.1049A>G, c.1349C>G, and c.1911G>A (examined in at least 200 Czech control chromosomes) were 0.56, 0.34, 0.14, 0.05, and 0.31, respectively.

PCR product, using the appropriate conditions, and were followed by electrophoresis in an agarose gel or in a 12% polyacrylamide gel. The mutations found in each patient were also analyzed in genomic DNA samples of both parents when available (Patients 2, 3, 4, 5, 7, 8, and 9); only a maternal DNA sample was available in Patient 6.

The previously described PCR-RFLP method for detection of the Iberian mutation c.1361C>T [Vilaseca et al., 2003] was inapplicable in the paternal sample of Patient 4 due to a presence of novel SNP c.1349C>G in the region for hybridization of the sense primer at the paternal wild-type allele. Therefore, sequencing of a PCR product, which was amplified with another pair of primers (5′CTGATTTAAGAGTAAGAAAATGC3′ and 5′TGTCTTCCATAAAGCAGGT3′), was employed to confirm the presence of pathogenic mutation c.1361C>T in the father of Patient 4.

In selected cases, separation of parental alleles for haplotype determination or verification of compound heterozygosity was achieved by amplifying the entire *MTRR* coding sequence in one segment using cDNA as a template (PCR primers: 5′GCGTTGTG CAGGTTCGTG3′ and 5′GAAGGGCGTAGATAGAAGATAC3′), and subsequent cloning into the pCR4-TOPO vector (Invitrogen). Plasmids were isolated using the Qiagen Plasmid Mini Kit (Qiagen) and the presence of mutations and polymorphisms was determined by RFLP analysis (c.66A>G and c.524C>T) or by direct sequencing (c.903+469T>C, c.1049A>G, c.1349C>G, c.1361C>T, and c.1573C>T).

Functional Studies

Immortalization of fibroblasts. Due to a consistently low transfection efficiency of untransformed human fibroblasts, immortalized cell lines were prepared as described below from the fibroblasts of six patients (Patients 1, 2, 3, 4, 5, and 7) and from one patient (Patient AX) with isolated functional methionine synthase deficiency but not belonging to the *cblE* or *cbl*G complementation group [Fowler et al., 1997b]. Attempts to immortalize fibroblasts of Patient 6 were unsuccessful and untransformed fibroblasts had to be used for functional studies.

Fibroblasts were immortalized using a plasmid pRNS1 containing SV40 DNA sequences [Litzkas et al., 1984]. Cells were plated in 12-well dishes and transfected when 80 to 90% confluent with 2 μg vector DNA and 4 μL Lipofectamine 2000 transfection reagent (Invitrogen) in a total of 1 mL serum and antibiotic-free Dulbecco's Modified Eagle medium (DMEM, Gibco). After 6 hr of incubation, the medium was replaced by DMEM supplemented with 10% FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma, www.sigmaaldrich.com). Cells were allowed to recover for 48 hr and then subcultured (split ratio of 1:3) in the same medium supplemented with 0.2 mg/mL geneticin (Invitrogen) to select for immortalized cells. After 10–14 days, colonies of rapidly dividing cells were separated, cultured to confluence and used for transfection studies.

Preparation of the MTRR minigene. The entire cDNA of the cytosolic isoform of *MTRR* (GenBank Accession No. AF025794.1) was amplified by PCR (PCR primers: 5′ATGAGGTTTCTGT3′ and 5′TTATGACCAAATATCCT3′) and inserted into the eukaryotic expression vector pcDNA4/HisMax-TOPO (Invitrogen) according to the manufacturer's instructions. The *MTRR* containing plasmid (*pMTRR*) was propagated in TOP10 One Shot cells, isolated using the Qiagen Plasmid Mini Kit (Qiagen), and amplification errors were ruled out by sequencing.

Transient transfection with pMTRR. For transfection experiments, the *pMTRR* plasmid containing the wild-type *MTRR* gene and the *pLacZ* plasmid containing the gene for β-galactosidase (pcDNA4/HisMax/*LacZ*; Invitrogen) were isolated using the Plasmid Endofree Maxi Kit (Qiagen). The transfection procedure was optimized attempting cationic lipid transfection using reagents FuGENE 6 (Roche, www.roche-applied-science.com), GenePORTER 2 (Gene Therapy Systems, www.genetherapysystems.com), Lipofectamine 2000 (Invitrogen) and SuperFect (Qiagen), respectively. Out of these, FuGENE 6 exhibited the highest transfection efficiency (data not shown). As cotransfection of cells with both *pMTRR* and *pLacZ* plasmids was associated with rather low levels of MTRR expression (data not shown), we transfected cells with either *pMTRR* or *pLacZ* plasmids in separate flasks.

One day before transfection, each culture of immortalized cells was split into three T25 cm^2 flasks so that they were 60 to 80% confluent at the time of transfection. One culture was transiently transfected with the *pMTRR* plasmid and two cultures were transfected with the *pLacZ* plasmid (2.4 μg plasmid DNA/culture) using 7.2 μL FuGENE 6 transfection reagent in a total of 5 mL serum and antibiotic-free DMEM, according to the manufacturer's instructions. After 4–6 hr incubation, the medium was replaced by DMEM supplemented with 10% FCS, 2 mmol/L L-glutamine, and antibiotic-antimycotic mixture. After further incubation for 2–3 days, when cells were almost confluent, the expression of the *MTRR* gene was monitored by measuring the formation of methionine in the *pMTRR* transfected culture using one *pLacZ* transfected culture as the blank. Transfection efficiency was monitored by assay of β-galactosidase activity in the second *pLacZ* transfected culture.

Statistical Analysis

The effect of the *MTRR* minigene expression on ^{14}C-methionine formation in *cblE* cells was evaluated by the two-sided paired two-sample *t*-test after testing the normality of distribution by the use of statistical software Prophet 5.0. (BBN Systems and Technologies, www.bbn.com) The tests were performed at a 5% level of significance.

RESULTS

Clinical and Biochemical Findings

The main clinical findings at diagnosis and at the time of the study are summarized in Supplementary Table S1 (www.interscience.wiley.com/jpages/1059-7794/suppmat). The observed sex ratio of six females and three males is in agreement with an autosomal recessive mode of transmission. In this group of patients, the age of appearance of clinical abnormalities ranged from 2 weeks to 3 years, with a median age of 4 weeks. However, despite this early manifestation, the age at which the diagnosis was established was much later, ranging from 4 weeks to 14 years (median 1.5 years). Main laboratory findings, enzyme data, and genotypes are shown in Table 1. The concentration of tHcy was elevated at diagnosis in all patients, ranging from 44 to 169 μmol/L (median 92). Plasma methionine levels varied between 5 and 27 μmol/L (median 16), with low values in only four patients (Patients 1, 6, 7, and 8).

Hemoglobin (Hb) levels at the time of first clinical manifestation were clearly reduced compared to age-related controls in all patients, while MCV values were clearly elevated only in Patients 4, 5, and 6. Bone marrow was investigated in Patients 2, 3, 4, 5, 6, 7, and 9, and all revealed megaloblastosis except Patient 9. Anemia responded to folate treatment only in Patient 7, details of whom have been reported elsewhere [Zavadakova et al., 2002]. Clinically manifested thromboembolic events were not observed in any patient. However, possible vascular system involvement was indicated by arterial hypertension in Patients 6 and 8.

Neurological involvement at diagnosis was judged to be severe in all but Patients 4 and 5, based on the presence of delayed

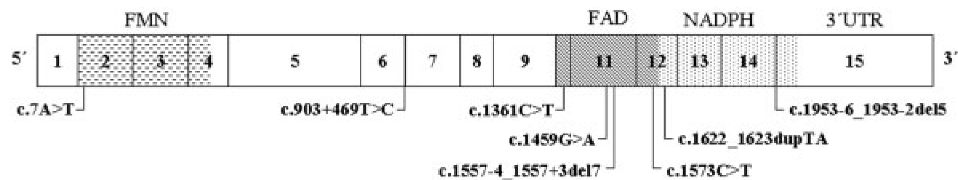

FIGURE 1. **Schematic representation of the methionine synthase reductase gene and localization of mutations identified within this study. White boxes represent exons; binding domains are depicted as a dashed box (FMN), shaded box (FAD), and dotted box (NADPH).**

development combined with various types of brain atrophy, abnormalities of brain imaging, and/or abnormal electrophysiological tests. Patients 4 and 5 showed no clinically apparent neurological impairment and their development was normal. Only small brainstem lesions and slight electrophysiological abnormalities were observed in Patients 4 and 5 at the ages of 14 and 10 years, respectively.

All patients were treated from the time of diagnosis with standard doses of hydroxocobalamin, betaine and folates. Length of treatment varied between 1 and 14 years with a median of 3.5 years, and no substantial adverse effects were observed during the total of 46 treatment-years. Moderate improvement of psychomotor development was observed in Patients 1 and 2, but not in Patients 6, 7, 8, and 9, while further deterioration occurred in Patient 3 despite receiving a standard therapy. Follow-up investigation of brain structural abnormalities was performed in six patients. No substantial improvement of brain atrophy or white matter changes on therapy was noted, with the exception of Patients 1 and 7, who showed regression of white matter abnormalities. In contrast, motor skills improved in Patients 1, 2, 3, 7, and 8. In summary, the clinical data in our study suggest at most a moderate effect of current treatment approaches on neurodevelopmental disabilities in patients with *cblE* defect.

The formation of methionine from formate in intact fibroblasts as an indirect measure of the activity of the methionine synthase/methionine synthase reductase complex was markedly reduced to less than 10% of the mean control value in all nine patients. The activity in the patients with normal development (Patients 4 and 5) was reduced to a similar extent to that seen in the other patients.

Somatic complementation analysis confirmed that all patients belong to the *cblE* complementation group. Thus, there was a clear increase of formation of methionine in fibroblasts from each patient when fused with the *cblG* cell lines, but not when fused with the reference *cblE* fibroblasts or after self-fusion (data not shown).

Mutation Analysis

Sequencing of the coding region of *MTRR* revealed mutations in both parental alleles in all nine patients (Table 1). Three mutations are novel, whereas five have been reported earlier (Table 2) [Wilson et al., 1999a; Zavadakova et al., 2002; Vilaseca et al., 2003]. Three mutations were missense (c.1361C>T, c.7A>T, and c.1459G>A), one mutation was a duplication (c.1622_1623dupTA), one was a nonsense mutation (c.1573C>T), and three mutations affected splicing (c.903+469T>C, c.1557-4_1557+3del7, and c.1953-6_1953-2del5). The genotypes of *cblE* patients and details of all identified sequence changes are summarized in Tables 1 and 2, and Figure 1. None of the identified mutations were present in 100 Czech control alleles, thereby substantially decreasing the possibility that these variants constitute common polymorphisms.

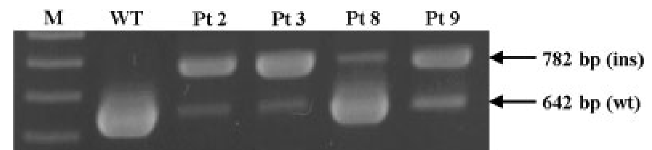

FIGURE 2. **Gel electrophoresis of RT-PCR fragments illustrating the normal and aberrant splicing due to c.903+469T>C mutation. This mutation activates the putative exon splicing enhancer and leads to an insertion r.903_904ins140 at the mRNA level. Amplification of the middle portion of *MTRR* cDNA yields a 642-bp product in control cDNA, while mutation r.903_904ins140 results in a 782-bp PCR product. *M*, molecular weight marker; *WT*, wild-type control; *Pt 2*, *Pt 3*, *Pt 8*, and *Pt 9*, Patients 2, 3, 8, and 9, respectively. Sufficient amount of each sample was loaded to allow detection of even small amounts of RT-PCR product. The presence of a small amount of normally spliced *MTRR* mRNA (642-bp fragment) in Patients 2 and 3, both homozygous for the r.903_904ins140 mutation, suggests incomplete missplicing. In Patient 8, heterozygous for this mutation, preferential amplification of the sequence from the mutant allele r.1462_1557del96 and/or instability of the mRNA from the mutant allele r.903_904ins140 are indicated. In Patient 9, also heterozygous for mutation r.903_904ins140, the apparent instability of the mRNA from the other mutant allele carrying c.1953-6_1953-2del5 is indicated.**

Analysis of genomic DNA samples available from parents of Patients 2, 3, 4, 5, 6, 7, 8, and 9 identified parental heterozygosity for the respective pathogenic mutations found in the patients. These findings indicated that Patients 6, 7, 8, and 9 are compound heterozygotes, and Patients 2, 3, 4, and 5 are homozygotes for the pathogenic mutations.

The insertion r.903_904ins140 was found in six *MTRR* alleles. Homozygosity for this mutation was observed in Patient 2, whose family history suggests possible consanguinity, and in Patient 3, born to consanguineous parents. In addition, Patients 8 and 9 were heterozygous for this insertion. The insertion leads to a frameshift, resulting in the formation of a premature termination codon (p.N302fsX362). Missplicing is putatively caused by activation of an exon splicing enhancer due to a T>C transition deep within intron 6 (c.903+469T>C) [Wilson et al., 1999a]. However, the extent of missplicing may be incomplete, as Wilson et al. [1999a] proposed, since RT-PCR analysis revealed production of small amounts of normally spliced *MTRR* mRNA in both homozygous patients (Fig. 2).

The point substitution c.1361C>T leads to a replacement of serine 454 by leucine and was observed in five independent alleles, either in the homozygous (Patients 4 and 5) or heterozygous (Patient 6) state. This mutation has so far only been identified in patients with Spanish or Portuguese ancestry, supporting the idea that this is an Iberian mutation [Vilaseca et al., 2003].

The remaining mutations were identified only in one or two alleles. The novel mutation c.7A>T, which replaces the third amino acid arginine by threonine, was identified in the homozygous state in Patient 1, who was born to consanguineous parents. The other mutations were found only in the compound heterozygous state. The previously published point substitution, c.1459G>A, leads to replacement of the highly conserved glycine 487 by arginine [Wilson et al., 1999a]. The known deletion r.1462_1557del96 is caused by aberrant splicing of exon 11 due to a 7-bp deletion within the exon 11/intron 11 junction (g.22116_22122del7; c.1557-4_1557+3del7) [Wilson et al., 1999a]. The resulting absence of a region of splice donor leads to utilization of an alternative splice donor upstream from the 3′-end of exon 11. At the protein level, the large deletion without a frameshift leads to the removal of 32 amino acids from the MTRR protein. The novel deletion c.1953-6_1953-2del5, within the intron 14/exon 15 boundary, destroys the splice acceptor and most likely leads to missplicing of this region. The impact of this mutation on mRNA structure is unknown, since we were unable to detect any transcript arising from the allele carrying this deletion. The duplication c.1622_1623dupTA leads to a frameshift and to a formation of a premature termination codon, while the novel transition c.1573C>T directly causes the replacement of arginine 525 by termination codon.

Analysis of *MTRR* Alleles With Predicted Instability Due To Nonsense-Mediated Decay

Impaired stability of mRNA molecules due to nonsense-mediated decay (NMD) [Culbertson, 1999] was suggested by the finding of decreased yield of RT-PCR product(s) in Patients 2, 3, 6, 7, 8, and 9 (Figs. 2 and 3), who are all carriers of mutations leading to formation of a premature termination codon (p.N302fsX362, p.R525X, and p.M542fsX542). Therefore, in order to determine the abundance of such *MTRR* mRNA molecules, we used a simple PCR-RFLP method, which allows comparison of the proportion of each parental allele at the genomic DNA or mRNA level, respectively. This method is applicable to those individuals who are heterozygous for both the nonsense mutation and for one of the known polymorphisms, which was the case only in Patients 6 and 7.

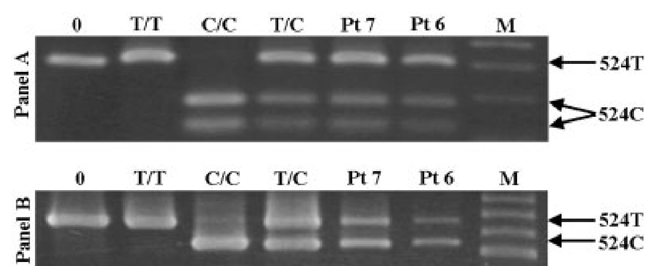

FIGURE 3. **Gel electrophoresis of PCR/RFLP fragments illustrating the relative abundance of mRNAs that carry premature termination codon. Panel A: Analysis of genomic DNA. Restriction analysis yields fragments of 304 bp in the presence of the T allele in position 524, and of 181 bp and 123 bp if the C allele is present. Panel B: RT-PCR/RFLP analysis of total fibroblast RNA. The presence of T and C at position 524 yields 642-bp and 518-bp fragments, respectively. *0*, nondigested control fragment; *T/T*, wild-type control c.524T; *C/C*, wild-type control c.524C; *C/T*, wild-type control c.524T/C; *Pt 7*, Patient 7 [c.1622_1623dupTA; c.524T] + [c.1459G>A; c.524C]; *Pt 6*, patient 6 [c.1573C>T ; c.524T] + [c.1361C>T; c.524C]; *M*, molecular weight marker. The results in Patients 6 and 7 suggest that mRNA molecules bearing the nonsense mutations c.1622_1623dupTA and c.1573C>T are present in concentrations similar to those mRNAs that carry the missense mutations c.1459G>A or c.1361C>T.**

Surprisingly, the yield of PCR-RFLP fragments, derived from the alleles carrying the nonsense mutations p.R525X or p.M542fsX542, was not decreased compared to those derived from the alleles with missense mutations (Fig. 3), suggesting that mRNAs carrying these two nonsense mutations were not subject to NMD. It remains possible that alleles carrying the missense mutations are also unstable, leading to degradation of both mRNA molecules in compound heterozygotes. Accurate quantification of mRNA would be needed to rule out this possibility.

Polymorphisms and Haplotype Analysis

Sequencing of patient samples revealed, in addition to the pathogenic mutations, five common genetic variants in the *MTRR* coding sequence. Four of these substitutions (c.66A>G, c.524C>T, c.1049A>G, and c.1349C>G) lead to an amino acid change (p.I22M, p.S175L, p.K350R, and p.P450R, respectively) while one (c.1911G>A) is a synonymous mutation (p.A637A). The prevalence of these variants determined in at least 200 Czech control alleles varied from 0.05 to 0.56 (for details see Table 2).

These polymorphisms were used for physical haplotyping of alleles carrying the most common pathogenic *MTRR* mutations (c.903+469T>C and c.1361C>T) to determine whether the mutant alleles originated from a single ancestral chromosome or arose independently. Haplotypes were determined in separated alleles using the following polymorphisms, c.66A>G–c.524C>T–c.1049A>G–c.1349C>G–c.1911G>A. The alleles carrying the intronic mutation (c.903+469T>C), which leads to the large insertion (r.903_904ins140), were associated with only one particular haplotype (G-C-A-C-G), supporting the idea that this mutation occurred in a single ancestral chromosome and was then distributed throughout Europe. In contrast, the Iberian mutation c.1361C>T was associated with three different haplotypes: G-**T**-A-C-**G**, G-**C**-A-C-**A**, and G-**C**-A-C-**G**. It is conceivable that the mutation occurred repeatedly by deamination of methylcytosine in different chromosomes, since the cytosine 1361 is located in a CpG dinucleotide. Alternatively, the mutation may have occurred only once on a chromosome G-C-A-C-G, and additional mutations in CpG dinucleotides 524 or 1911 subsequently formed the haplotypes G-**T**-A-C-G or G-C-A-C-**A**.

Functional Studies

Functional studies were aimed at proving that defects in the *MTRR* gene are indeed responsible for the *cblE* type of homocystinuria. In parallel cultures of *cblE* cells transfected with either *pMTRR* or *pLacZ*, we examined the restoration of methionine synthase activity indirectly by measuring the formation of ^{14}C-methionine in cells incubated with ^{14}C-formate. Functional studies were performed using immortalized fibroblasts, with the exception of Patient 6.

Figure 4 shows results of transfection experiments in seven *cblE* cell lines and in cells of the Patient AX, with isolated functional methionine synthase deficiency but not belonging to the *cblE* or *cblG* complementation group, as control. Results of all experiments showed an approximately four-fold increase in formation of methionine after transfection with the *pMTRR* plasmid (mean=0.57 nmol/16 hr/mg protein) compared with activity after transfection with the *pLacZ* plasmid (mean=0.13 nmol/16 hr/mg

protein). Significance of this increase was confirmed by paired two-sample *t*-test ($P < 0.001$). Transfection was performed on two to four occasions with each cell line, with a reproducibly enhanced methionine synthesis in each cell line and in each experiment, albeit to a varying degree (2–33 times). This large variation is in part explained by variation in transfection efficiency, as shown by the linear relationship between the increase in formation of methionine and the logarithm of β-galactosidase activity determined in parallel cultures (Fig. 5). In contrast, no clear increase in formation of methionine in fibroblasts of Patient AX (with isolated functional methionine synthase deficiency but not belonging to the *cblE* or *cblG* complementation group) was observed following transfection with the *pMTRR* plasmid (Fig. 4), thus demonstrating that the effect of *pMTRR* expression on *cblE* cell lines is specific.

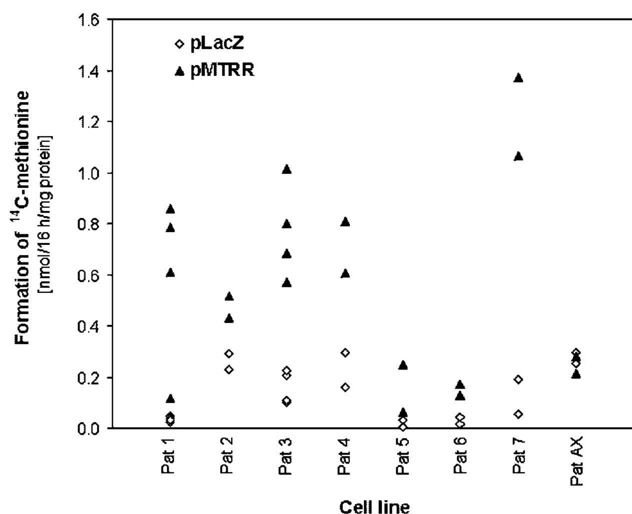

FIGURE 4. **Expression of the wild-type *pMTRR* minigene construct in cells of seven *cblE* patients and in cells of Patient AX (with isolated functional methionine synthase deficiency but not belonging to the *cblE* or *cblG* complementation group) as control. Formation of ^{14}C-methionine from ^{14}C-formate was assayed in cells after transfection with the *pMTRR* vector (▲) and *pLacZ* vector (◇) as the blank, as described in the section Materials and Methods. Values represent single determinations in several independent experiments. Cells were immortalized fibroblasts except for those of Patient 6, which were untransformed fibroblasts.**

DISCUSSION

One of the aims of our study was to examine by wild-type *MTRR* minigene expression whether defects in the *MTRR* gene are indeed responsible for the *cblE* type homocystinuria. MTRR is one of two proposed reducing systems activating methionine synthase, and identification of mutations in this gene in 13 *cblE* patients supports its role in the pathogenesis of *cblE* [Leclerc et al., 1998; Wilson et al., 1999a; Zavadakova et al., 2002; Vilaseca et al., 2003]. However, direct evidence of pathogenicity of the various *cblE* mutant alleles is lacking. In our study transfection of cell lines from seven patients with the *cblE* defect with the wild-type *MTRR* minigene led to significant increase in formation of methionine in all of them, indicating restoration of methionine synthase activity. In contrast, we did not observe any increase of methionine formation in cells of patient with isolated functional methionine synthase deficiency but not belonging to either the *cblE* or *cblG* complementation group. Although functional methionine synthase deficiency may be caused by defects in other genes, our study provides direct functional evidence that the *cblE* type of homocystinuria is indeed caused by defects in the *MTRR* gene.

Our study increases knowledge of the spectrum of mutations in the *MTRR* gene in *cblE* patients. In nine patients of European origin, we identified eight different mutations. Several lines of evidence support the pathogenicity of these mutant alleles. First, the absence of any of these mutations in 100 control alleles suggested that they are not common polymorphisms. Second, identified transitions, c.1361C>T and c.1459G>A, lead to a substitution of amino acid residues 454 and 487, which are highly conserved between human and *C. elegans* MTRR and human cytochrome P450 reductase, and occur in the putative FAD binding domain. Third, three identified mutations (c.903+469T>C, c.1573C>T, and c.1622_1623dupTA) result in formation of premature termination codons. Translation of such

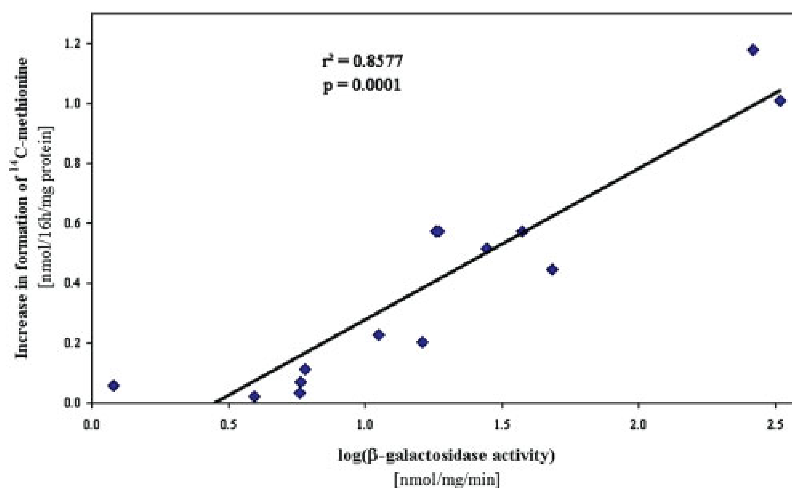

FIGURE 5. **Correlation between the expression of the wild-type *MTRR* minigene and transfection efficiency in cells of seven *cblE* patients. Expression of *MTRR* is shown as the formation of ^{14}C-methionine in cells transfected with *pMTRR* minus the value obtained in cells transfected with *pLacZ*. Transfection efficiency was estimated by measuring β-galactosidase activity after transfection with the *pLacZ* vector. Values represent single determination in two independent experiments. For further details see the Materials and Methods section and the legend for Figure 4.**

mRNAs may produce truncated mutant proteins lacking FAD or NADPH binding domains, or the mRNAs may be unstable due to NMD. However, we found no evidence of NMD in two analyzed mutations (c.1573C>T and c.1622_1623dupTA). Fourth, the large deletion r.1462_1557del96 leads to a removal of the C-terminal part of the FAD binding domain and is highly likely to lead to loss of function. Fifth, the mRNA produced from the c.1953-6_1953-2del5 mutant allele is very unstable and pathogenicity is probably due to lack of mRNA molecules for translation.

The mutation r.903_904ins140, resulting from activation of an exon splicing enhancer due to a T>C transition within intron 6, has already been reported in four alleles in three unrelated patients [Wilson et al., 1999a; Zavadakova et al., 2002]. Findings in our study extend the number of r.903_904ins140 alleles to eight, so that this insertion is so far the most common pathogenic mutation in the *MTRR* gene. Furthermore the hypothesis of Wilson et al. [1999a] that the missplicing might be incomplete is also supported by our observation that some normally spliced mRNA is produced in two homozygous patients (Patients 2 and 3). We previously suggested that small amounts of normal *MTRR* mRNA may be sufficient for synthesis of some active MTRR protein, leading to the favorable clinical outcome in the homozygous Patient 2 [Zavadakova et al., 2002]. However, this is not supported by the presence of severe neurological damage in Patient 3. It is more likely that the favorable clinical presentation in Patient 2 compared to Patient 3 is due to early diagnosis and treatment.

Only a few detailed reports on clinical and biochemical features of *cblE* patients have been published to date [Watkins and Rosenblatt, 1989; Fowler et al., 1997a; Zavadakova et al., 2002; Vilaseca et al., 2003]. Our observations in nine *cblE* patients of European origin provide new information on the clinical and biochemical phenotype of this disorder, as follows. First, hypomethioninemia appears to be an inconsistent finding, being present in only four of these patients. Second, the anemia in *cblE* patients is not constantly associated with macrocytosis, since only three patients showed increased MCV at the time of initial investigation. However, in those seven patients in whom bone marrow was examined, megaloblastic changes were lacking in only one. Third, it appears that major nervous system involvement is not always present, although it has to be borne in mind that the appearance of neurological impairment may be obscured by the age of diagnosis and by introduction of treatment. Nevertheless, it is clear that two patients lacked the severe neurological involvement seen in the other seven patients and those reported previously [Vilaseca et al., 2003]. It is possible that such patients may remain undiagnosed, and therefore, measurement of tHcy in all patients with unexplained macrocytic anemia, even in the absence of neurological involvement, is recommended. Fourth, no clinically manifested thromboembolic events were noted in this group of patients during 86.5 patient-years, of which 46 patient-years were on treatment. However, an association between the *cblE* defect and vascular abnormalities cannot be excluded, since the incidence of vascular events was 1 in 25 patient-years in patients with other types of hyperhomocysteinemia, who have substantially higher homocysteine levels [Mudd et al., 1985; Yap et al., 2001].

Genotype–phenotype correlation is difficult in such a small group of patients, and conclusions can only be reliably made in patients who are homozygous for a single pathogenic mutation. In our study, homozygosity for the Iberian mutation c.1361C>T was found in the two mildly affected patients without severe neurological involvement, while homozygosity for mutations c.7A>T or c.903+469T>C was presented in three patients with white matter abnormalities at the time of diagnosis. The severe neurological consequences of the latter mutation seem to be preventable by early treatment as demonstrated by substantially different intelligence quotients (IQs) of Patients 2 and 3, who differ only in the time when therapy was started (Supplementary Table S1). Nevertheless, early treatment may not prevent severe neurological sequelae in patients with other genotypes, as demonstrated in Cases 8 and 9 (Supplementary Table S1).

Clear conclusions on the benefit of therapy in the *cblE* type of homocystinuria cannot be made, due to the small numbers of patients, genetic heterogeneity, the variable age of diagnosis, start and duration of treatment, and finally the lack of standardized investigation of outcome measures.

In summary, identification of mutations in fibroblasts of nine European *cblE* patients, and correction of impaired methionine synthase activity by the *MTRR* minigene, confirmed the hypothesis that defects in methionine synthase reductase are the cause of the *cblE* type of homocystinuria.

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