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Molecular basis for methionine synthase reductase deficiency in patients belonging to the *cblE* complementation group of disorders in folate/cobalamin metabolism

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Methionine synthase reductase (MSR) deficiency is an autosomal recessive disorder of folate/cobalamin metabolism leading to hyperhomocysteinemia, hypomethioninemia and megaloblastic anemia. Deficiency in MSR activity occurs as the result of a defect in the MSR enzyme, which is required for the reductive activation of methionine synthase (MS). MS itself is responsible for the folate/cobalamin-dependent conversion of homocysteine to methionine. We have recently cloned the cDNA corresponding to the MSR protein, a novel member of the ferredoxin-NADP$^+$ reductase (FNR) family of electron transferases. We have used RT–PCR, heteroduplex, single-strand conformation polymorphism (SSCP) and DNA sequence analyses to reveal 11 mutations in eight patients from seven families belonging to the *cblE* complementation group of patients of cobalamin metabolism that is defective in the MSR protein. The mutations include splicing defects leading to large insertions or deletions, as well as a number of smaller deletions and point mutations. Apart from an intronic substitution found in two unrelated patients, the mutations appear singular among individuals. Of the eleven, three are nonsense mutations, allowing for the identification of two patients for whom little if any MSR protein should be produced. The remaining eight involve point mutations or in-frame disruptions of the coding sequence and are distributed throughout the coding region, including proposed FMN, FAD and NADPH binding sites. These data demonstrate a unique requirement for MSR in the reductive activation of MS.

INTRODUCTION

Methionine synthase (MS; EC 2.1.1.13) catalyzes the cobalamin (vitamin B_{12})-dependent methylation of homocysteine to form methionine using 5-methyltetrahydrofolate as the methyl donor (Fig. 1A) (1,2). Over time, the highly reactive cobalamin(I) cofactor of MS is oxidized to the inert cobalamin(II) form, rendering the enzyme inactive (3). MS must then be reactivated through reductive methylation using *S*-adenosylmethionine as the methyl donor. We recently cloned the cDNA corresponding to the protein responsible for this reactivation, and called the enzyme methionine synthase reductase (MSR; EC 2.1.1.135) (4). MSR is a novel member of the ferredoxin-NADP$^+$ reductase (FNR) family of electron transferases, containing putative FMN, FAD and NADPH binding sites necessary to maintain MS in its functional state (Fig. 1B). The enzyme contains 698 amino acids and has a predicted molecular weight of 77 000 Da (4). A predominant mRNA of 3.6 kb was detected by northern blot analysis. The gene coding for MSR, *MTRR*, has been localized to chromosome 5p15.2–p15.3 (4).

Functional deficiency of MS is an autosomal recessive disorder of folate/cobalamin metabolism (5,6). Two forms of the disease have been described, each the result of a defect in a single gene product as defined through cell fusion-based complementation studies (7). One form occurs as the result of a defect in the MS protein itself and is defined by the *cblG* complementation group of disorders in cobalamin metabolism. The second form is defined by the *cblE* complementation group, corresponding to a deficiency in MSR. Both enzymatic defects lead to hyperhomocysteinemia, homocystinuria, hypomethioninemia and megaloblastic anemia without methylmalonic aciduria (5,6). Patients exhibit a range of clinical symptoms including severe developmental delay, ataxia, cerebral atrophy, neonatal seizures and blindness. Initial studies of mutations in *cblE* patients were used to authenticate the MSR cDNA clone (4). Two mutations were identified, one a 4 bp deletion resulting in a frameshift and the other the in-frame deletion of a highly conserved leucine residue. In this study, we have continued the analysis of mutations in *cblE* patients. We now report the identification of eleven new mutations, including splice defects leading to large insertions or deletions in the mRNA as well as a number of smaller deletions and point mutations. The presence of these mutations in the *MTRR* gene of patients deficient in the reductive activation of MS indicate that the MSR protein is required for the proper function of the MS enzyme.

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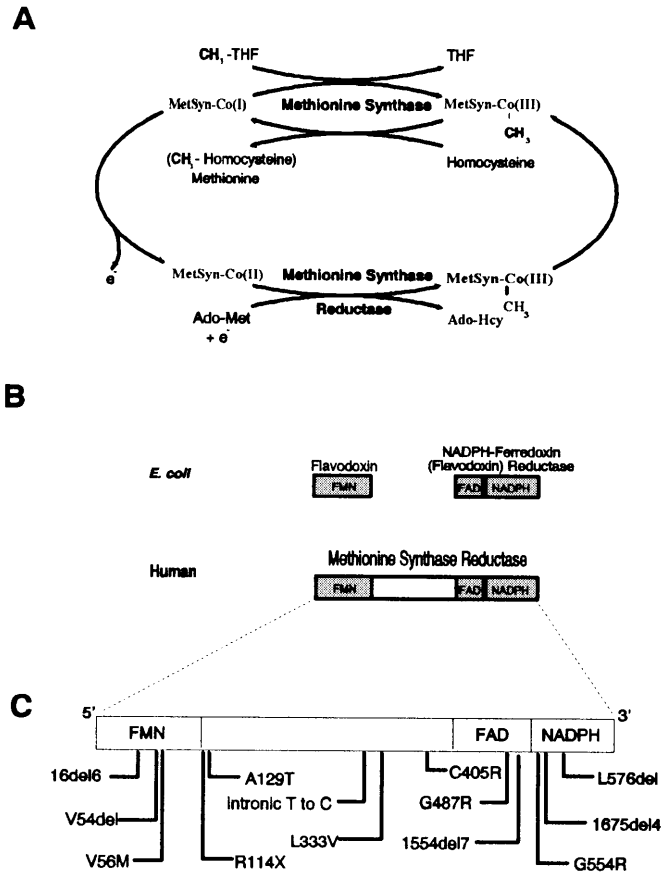

Figure 1. Role, structure and location of mutations in methionine synthase reductase. (**A**) Overall reaction of methionine synthase and methionine synthase reductase, involving the transfer of a methyl group from methyltetrahydrofolate (CH_3-THF) to homocysteine using methionine synthase–methylcobalamin [MetSyn–CH_3-Co(III)] as the intermediate methyl carrier. The reductive methylation in the lower part of the scheme is the mechanism by which methionine synthase reductase uses *S*-adenosylmethionine (Ado-Met) together with an electron to reactivate MS after oxidative inactivation. Ado-Hcy, *S*-adenosylhomocysteine. (**B**) Enzymes involved in the reduction of methionine synthase. The upper scheme shows the two-flavoprotein system involved in the reductive activation of cobalamin-dependent methionine synthase in *E.coli*. FMN and FAD/NADPH binding sites are in different proteins. The lower scheme shows MSR, with all of the binding sites necessary for MS activation fused in a single protein. (**C**) Schematic of MSR showing the 11 new mutations as well as the two previously reported mutations (1675del4 from WG1146/WG788 and L576del from WG1836) in relation to FMN, FAD and NADPH binding sites.

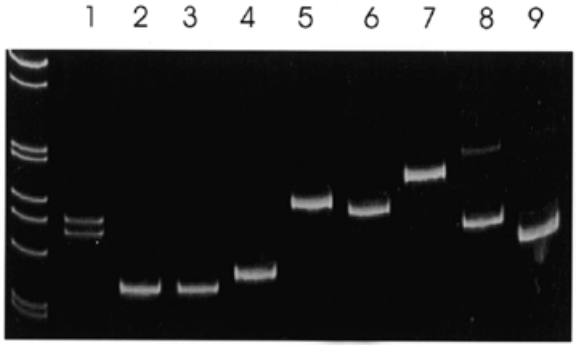

Figure 2. Acrylamide gel showing PCR segments used for mutation analysis. The MSR cDNA was divided into nine overlapping segments of 250–450 bp in length. Lane 1 shows two PCR products due to alternative splicing. The faint upper band in lane 8 is a consistent artifact unrelated to MSR. Segment lengths, primers used, and annealing temperatures are described in Table 1. The lane at the far left shows molecular weight markers.

Table 1. cDNA sequence segments used in heteroduplex and SSCP analysis[a]

Segment number[b]	Nucleotide position[c]	Primer pairs[d]	Product length (bp)[e]
1	–79–278	*AB191/*1803E	357
2	184–431	*AB581/*1103A	247
3	335–584	*1902C/AB582	249
4	506–760	*Z596/*Z597	254
5	673–1061	AB583/AB584	388
6	982–1369	AB585/*Z594	387
7	1318–1766	*Z116/*Z117	448
8	1663–1998	*AB586/*AB588	335
9	1882–2173	AB587/*2101C	291

[a]Annealing to generate each segment was carried out at 62°C.
[b]Number of PCR segment.
[c]Numbering of nucleotide position counted from the A of the ATG initiation codon (4).
[d]Sense/antisense oligonucleotides used to generate each segment. AB582, 5′-CTCAGAAGCTCGACTTGAGATTC; AB583, 5′-GAGTCCTCACTTACCCGTTCGGTA; AB584, 5′-AGCTCCTTTCTTCTTTGTGTCTGC; AB585, 5′-CTCCAAAGACTGCAGCTTGAAGAT; AB587, 5′-AACATCCAGCTTCATGGCCAGCAG.
[e]Expected size of PCR product.
*Sequences given by Leclerc *et al*. (4).

RESULTS

The MSR cDNA was divided into nine overlapping segments of 250–450 bp in length in order to allow for RT–PCR dependent heteroduplex and single strand conformation polymorphism (SSCP) analysis of relatively small fragments of cDNA (Fig. 2; Table 1). Two splice variants, previously reported (4) and characteristic of all normal and mutant cell lines analyzed, were found near the 5′ end of the MSR cDNA which could complicate the detection of heteroduplexes. The first, resulting in the deletion of 154 bp at nucleotides 130 to 283, was avoided by placing sense and antisense primers within the deleted region so as to examine only cDNA containing the complete sequence (PCR segments 1 and 2).

The second, generating a 26 bp deletion at nucleotides –52 to –27, was not corrected for. This deletion makes two species of wild-type cDNA available for amplification, resulting in fragments of 331 and 357 bp and detected as a doublet in PCR segment 1 (Fig. 2, lane 1). This segment, when denatured and allowed to re-anneal, produced the expected heteroduplex pattern, represented as the middle two bands in Figure 3I, lane 1, as well as two unexpected upper bands. When the six bands present on acrylamide (Fig. 3I, lane 1) were purified and re-amplified, the original 331 and 357 bp fragments were regenerated, indicating that all six bands were the result of a

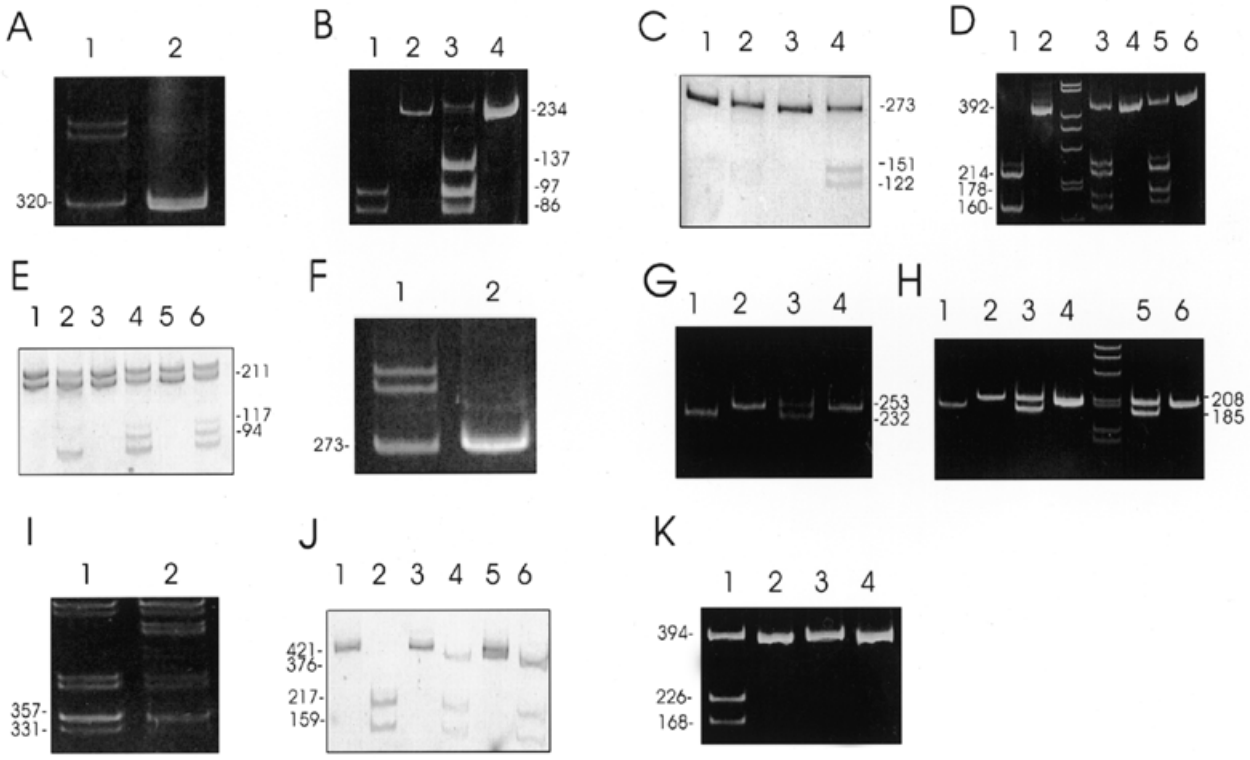

Figure 3. DNA or cDNA diagnostic tests for mutations in the MSR gene run on acrylamide gels. Details are given in Table 2. Tests were performed on genomic DNA unless otherwise stated. (**A**) V54del (in cDNA): lane 1, WG1384; lane 2, normal. (**B**) A129T: lane 1, normal digested; lane 2, normal undigested; lane 3, WG1384 digested; lane 4, WG1384 undigested. (**C**) G487R: lane 1, normal undigested; lane 2, normal digested; lane 3, WG2366 undigested; lane 4, WG2366 digested. (**D**) G554R: lane 1, normal digested; lane 2, normal undigested; lane 3, WG2366 digested; lane 4, WG2366 undigested; lane 5, control containing the silent polymorphism1553 G→A, digested; lane 6, undigested. (**E**) Intronic T→C: lane 1, normal undigested; lane 2, normal digested; lane 3, WG2317 undigested; lane 4, WG2317 digested; lane 5, 1296 undigested; lane 6, digested. (**F**) 1554del7: lane 1, WG1296; lane 2, normal. (**G**) R114X: lane 1, normal digested; lane 2, normal undigested; lane 3, WG2317 digested; lane 4, WG2317 undigested. (**H**) V56M: lane 1, normal digested; lane 2, normal undigested; lane 3, WG1146 digested; lane 4, WG1146 undigested; lane 5, WG788 digested; lane 6, WG788 undigested. (**I**) 16del6 (in cDNA): lane 1, normal; lane 2, WG1401. The multiple doublets in the control are the result of heteroduplex analysis on segment 1, previously mentioned to contain two PCR products. Doublets not found in control are the result of the mutation. (**J**) L333V: lane 1, normal control undigested; lane 2, normal digested; lane 3, WG1401 undigested; lane 4, WG1401 digested; lane 5, control containing mutation, undigested; lane 6, control containing mutation, digested. (**K**) C405R: lane 1, WG1575 digested; lane 2, WG1575 undigested; lane 3, normal digested; lane 4, undigested. Images (C), (E) and (J) have been inverted to allow for easier viewing of bands on background.

single PCR product and showing that the unusual upper bands were not the result of larger segment contamination. Additional experiments, including electrophoresis of the serial dilution of the PCR products, suggested that the two upper bands in Figure 3I correspond to single stranded secondary structures. The remaining PCR products, segments 2–9, behaved as single bands during electrophoresis in agarose and acrylamide gels, except for segment 8, which sometimes produced a faint upper band unrelated to MSR when the product was analyzed on an acrylamide gel (Fig. 2, lane 8).

The following paragraphs summarize the mutations identified in patient fibroblasts (see Table 2 for details of the diagnostic procedures used to confirm mutations). In patient WG1384, a complex heteroduplex pattern in segment 1 was found to be due to a deletion of 3 bp, 160delGTT (V54del). The mutation was identified through cDNA sequencing of the PCR products yielding the heteroduplex. The mutation was confirmed to be heterozygous in genomic DNA from WG1384, also by heteroduplex analysis and using primers designed to exclude the previously mentioned 26 bp deletion (Fig. 3A). The patient's second mutation was seen as an extra band during SSCP analysis of segment 2. cDNA sequencing using primer pairs AB581 and 1103A revealed that the extra band was caused by a point mutation, 385 G→A (A129T). The mutation eliminates one of two *Nsp*I restriction sites present in the normal 234 bp genomic segment amplified using an intronic sense primer and an exonic antisense primer (Fig. 3B).

In patient WG2366, a complex pattern containing multiple extra bands was detected through SSCP analysis of segment 7. One of the extra bands proved to be caused by a point mutation, 1459 G→A (G487R), and was identified through cDNA sequencing using primers Z116 and Z117. The mutation creates a single *Ear*I cut site not present in the normal sequence (Fig. 3C). A second extra band in segment 7 proved to be caused by another missense mutation, 1660 G→A (G554R). This mutation was identified through direct sequencing of the cDNA from segment 7. The mutation destroys one of two *Bsl*I sites in a 392 bp genomic segment amplified using intronic primer pairs (Fig. 3D). Unexpectedly, WG2366 also showed a band running above the 214 bp fragment. This band is most likely the result of incomplete digestion between the 214 bp fragment and the 18 bp fragment, resulting in a fragment of 232 bp. WG2366 is heterozygous for both mutations.

Table 2. Strategy for PCR-based diagnostic tests of mutations identified in the MSR enzyme

Designation[a]	Cell line (WG)	Mutation	Primers used[b]	Expected size of fragment	Restriction enzyme used	Expected size after digestion (bp)	
						normal	mutant
A	1384	V54del[c] (160delGTT)	AK45/AK46	208	–	heteroduplex	
B	1384	A129T (385 G→A)	1902C/AK43	234	*Nsp*I	97,86,51	137,97
C	2366	G487R (1459 G→A)	AI521/AI373	273	*Ear*I	273	151,122
D	2366	G554R (1660 G→A)	AK47/AK48	392	*Bsl*I	214,160,18	214,178
E	1296,2317	intronic T→C	AK55/AI233	211	*Hpa*II	211	114,97
F	1296	1554del7	AI521/AI233	273	–	heteroduplex	
G	2317	R114X (340 C→T)	AK43/AK44	253	*Acc*I	232,21	253
H	788, 1146	V56M (166 G→A)	AK45/AK46	208	*Rsa*I	185,23	208
I	1401	16del6[c]	AB191/1803E	357	–	heteroduplex	
J	1401	L333V (997 C→G)	AK52/AK54	421	*Bbv*I	217,159,45	376,45
K	1575	C405R (1213 T→C)	AK49/AK51	394	*Fsp*I	394	226,168

[a]According to Figure 2.

[b]Oligonucleotides used to amplify each fragment from 5′ to 3′: AK45, ctagtcactggactggtcgtctcaa; AK46, CTCCGGTGCCCGTGGTAGAAAGTA; 1902C (see ref. 4); AK43, gtaaatcaatctctggctgaccctctt; AI521, gtactgatatcgagttcaaaactt; AI373, gagtatgagttaacaatgctgagg; AK47, gtgacaaattatgttatcgcacatt; AK48, ctgatgccactatttagtgatgtt; AK55, catcttgccattttattcctga; AI233, gtggacagcttcctttcctgggcc; AK44, AATGGGGGGAAGATAATTGGTATA; AB191 (see ref. 4); 1803E (see ref. 4); AK52, gtatgcctgtgttcccggacctt; AK54, ctgagttcacatattaaaatcata; AK49, cataggatacaaagggtaagacaa; AK51, cttgggtaatgggtgcatcccta. Lower case letters indicate intronic sequences and the uppercase letters indicate exonic sequences; underlined letters indicate that the nucleotides were altered to create a new restriction site.

[c]RT–PCR was used to amplify the region with the mutation.

During diagnostic testing, one of the 50 control alleles also appeared positive for the mutation (Fig. 3D, lane 5). Upon sequencing, however, it was found that the control did not contain this mutation, but a silent change, 1653 G→A. Although this silent change should not have effected the *Bsl*I cut site, being found in a part of the recognition sequence that does not require a specific nucleotide, it did prove to affect cleavage so that it became ambiguous with the diagnostic test for 1660 G→A. Although 1653 G→A is rare, found in only one of the 50 control alleles tested, sequencing of samples that appear positive for the diagnostic test may be necessary to eliminate those containing the silent change.

In patients WG1296 and WG2317, an unexpected doublet was observed in agarose gel electrophoresis of amplified segment 5, which normally runs as a single band (data not shown). When excised and subcloned, the band running above the normal was found to contain an insertion of 140 bp of intronic DNA between nucleotides 903/904 of the cDNA sequence. Although the insertion itself does not contain a stop codon, it does change the reading frame of the cDNA, creating a downstream termination signal. The insert comes from a segment of DNA located well within the intron beginning after nucleotide 903. Sequencing of the insert led to the discovery of a point mutation, the substitution of a C for a T, 23 bp into the insert sequence. The mutation was confirmed by testing for the generation of an *Hpa*II restriction site in a 211 bp genomic segment generated using intronic primer pairs (Fig. 3E). Because the initial PCR reaction was smeared, the diagnostic test required excision and re-amplification of the PCR product. Re-amplification produced a doublet that could not be resolved and which produced a lower running band when digested (Fig. 3E, lanes 2, 4 and 6). These extra bands did not interfere with the diagnostic test as they were present in all samples tested. The mutation was found to be heterozygous in both WG1296 and WG2317.

In patient WG1296, an unexpected doublet was observed in agarose gel electrophoresis of amplified segment 7, which normally runs as a single band (data not shown). The band running below the normal was found to contain a deletion of 96 bp after nucleotide 1461. It was the result of a 7 bp deletion, 1554del7, identified through genomic sequencing of this region using intronic primer pairs to amplify a segment of 273 bp. Because the mutation is heterozygous, this fragment was used to confirm the mutation through heteroduplex analysis (Fig. 3F). This mutation results in the deletion of the natural 5′ donor splice site following nucleotide 1557, favoring the use of a new 5′ donor site 96 bp upstream of the original site. The new splice site results in the deletion of nucleotides 1462–1557 and the loss of 32 amino acids from the protein sequence, including a portion of the predicted FAD binding domain.

In patient WG2317, an extra band was detected during SSCP analysis of segment 2. The band was found to be the result of a nonsense mutation, 340 C→T (R114X), identified though cDNA sequencing. The mutation was confirmed using a modified sense primer to create a single *Acc*I site in the wild-type sequence, generating fragments of 232 and 21 bp in a 253 bp segment of DNA (Fig. 3G). WG2317 proved to be heterozygous for this mutation.

It was possible that the intronic T→C in WG1296 and WG2317 would be compatible with the generation of some normal transcript. This was evaluated on WG1296 because its two mutations were close enough to be amplified in one PCR product. Primers were made to prevent the amplification of the mutant alleles. The first was made to overlap the entry site of the 140 bp insert at position 903/904 so as to disrupt binding if the insert was present. The second was placed within the sequence defined by the 96 bp deletion so as to prevent ampli-

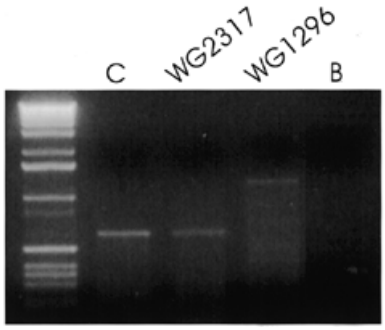

Figure 4. Agarose gel showing amplification of a 629 bp segment spanning the region between mutations in WG1296. The expected band is amplified in the control (C) and WG2317, but not in WG1296, which shows the amplification of a larger, unrelated band and smearing. B, water blank.

fication of the other mutant allele. While control cDNA showed amplification of the expected PCR product, it was undetectable in cell line WG1296 (Fig. 4). Instead, non-specific annealing of the primers produced a larger, unidentified product and additional smearing. WG2317 was also amplified using these primers. Since the cell line is heterozygous for only the 140 bp insertion, a product of intermediate intensity was observed as expected (Fig. 4). A second PCR reaction, amplifying an uninvolved region of the cDNA was performed simultaneously to ensure that the cDNA prep for WG1296 was not degraded. From these experiments, it can be observed that if any normal transcript does exist, it is at a level too low to be detected by PCR.

In siblings WG1146 and WG788, an extra band detected during SSCP analysis of segment 1 proved to be caused by a point mutation 166 G→A (V56M), identified through cDNA sequencing and confirmed through the loss of an artificially created *Rsa*I site in a 208 bp genomic segment (Fig. 3H). V56M is the second allele to be identified in these individuals, the first previously reported as a 4 bp deletion, 1675del4 (4). Both mutations were heterozygous in the affected siblings.

In patient WG1401, a complex heteroduplex pattern found in segment 1 led to the discovery of a 6 bp deletion, 16del6, which removes two leucine residues at amino acid positions 6 and 7. It was identified through the subcloning and sequencing of a 357 bp cDNA fragment generated using primer pairs AB191 and 1803E. The mutation was confirmed through heteroduplex analysis (Fig. 3I, lane 2). The complex heteroduplex pattern found within the control (Fig. 3I, lane 1) was due to the previously mentioned 26 bp deletion found to be associated with normal mRNA. The detection of an extra band during SSCP analysis in segment 5 of the same patient was found to be the result of a second mutation, 997 C→G (L333V), identified through direct sequencing of a cDNA segment generated using primer pairs AB583 and AB584. The mutation destroys one of two naturally occurring *Bbv*I restriction sites in a genomic segment 421 bp in length (Fig. 3J). During diagnostic testing, one of the 50 control alleles also appeared positive for the mutation (Fig. 3J, lane 6). A second diagnostic designed to use *Fnu4HI* also indicated the presence of the mutation in the control (data not shown). Sequencing confirmed that 997 C→G is present in the control sample, indicating that the mutation is an infrequent polymorphism. No other mutations were suggested through heteroduplex or SSCP analysis of the cDNA.

In patient WG1575, an extra band was discovered during SSCP analysis of segment 6. The band was caused by a point mutation, 1213 T→C (C405R), identified through the direct sequencing of a segment of cDNA generated using primers AB585 and Z594. The mutation was confirmed using a diagnostic test based on the fact that the mutation creates a single *Fsp*I site in a 394 bp segment of genomic DNA (Fig. 3K). WG1575 was found to be heterozygous for the mutation. The second allele has yet to be identified.

For each of the mutations resulting in a nucleotide substitution and where the result did not lead to a stop codon, 20–25 control samples were analyzed for mutations to ensure that they were not common polymorphisms. As mentioned above, 997 C→G was found in one of 50 control alleles.

DISCUSSION

Methionine synthase reductase deficiency was first characterized biochemically in patients possessing a defect in the enzyme required for the reactivation of methionine synthase (8,9). Cell fusion based complementation studies were used to assign these patients to the *cblE* complementation group of cobalamin disorders, distinguishing them from those patients possessing a deficiency in the MS enzyme itself and suggesting that two distinct proteins were involved in the conversion of homocysteine to methionine (5,7). The recent cloning of the MSR cDNA has allowed for the screening and identification of mutations in patient cell lines possessing a defect in the reductive activation of MS. The discovery of mutations in eight of nine families analyzed [including two from the previous study (4)] confirms the original complementation studies linking MSR to MS activation and provides a molecular explanation for the distinct *cblE* complementation group.

Structurally, the MSR protein represents a fusion of the two *E.coli* proteins required for the reductive activation of the cobalamin-dependent MS in that organism (Fig. 1B). MSR contains an FMN domain, corresponding by sequence homology to flavodoxin, and FAD and NADPH domains corresponding to flavodoxin reductase. As such, a single protein appears to account for reductive activation of MS in humans. Indeed, our identification of mutations in all three of the FMN, FAD and NADPH domains suggests the essential nature of all three components to the reactivation of MS, although whether the mutations affect the integrity of the resultant protein has yet to be determined.

Banerjee and colleagues (10,11) have proposed a two component protein system required for MS activation *in vitro*. One component is a soluble form of cytochrome b_5. The second component, located in the microsomal fraction of cells, could be replaced by cytochrome P450 reductase in the *in vitro* assays. The latter enzyme also contains independent FMN, FAD and NADPH binding sites and is 38% identical with MSR. The authors suggest that the reactivation of MS is mediated by cytochrome P450 reductase and that the soluble cytochrome b_5 affords a bridging function between the microsomally bound enzyme and cytoplasmic MS. They suggest further that multiple enzyme systems may be capable of activating MS which might allow for both P450 reductase

and MSR to show such activity (11). Our results do not support this interpretation. The very discovery of a severe genetic disease associated with MSR deficiency confirms the essential, unique requirement for this enzyme. In addition, the failure to identify *cblE* mutations in P450 reductase during the original cloning effort led us to reject this enzyme as being responsible for MSR deficiency. Furthermore, both *cblE* and *cblG* (primary MS deficiency) show a similar spectrum of disease severity, with the most severe disease compatible with life but with severe neurological involvement (12). If P450 reductase could substitute for MSR *in vivo*, a much milder, if not asymptomatic disease would be expected in *cblE* patients. There is no suggestion of a milder clinical phenotype in *cblE* disease. In fact, the two diseases are considered indistinguishable except through biochemical or complementation studies (7). Lastly, a reported case of cytochrome b_5 deficiency did not have any of the clinical or biochemical manifestations of *cblE* patients (13).

Then how does one explain the capacity of P450 reductase to activate MS *in vitro*? Flavodoxin and flavodoxin reductase have been shown to combine to support the 17a-hydroxylase activity of recombinant bovine cytochrome P450 17a-hydroxylase expressed in *E.coli* (14). Moreover, direct binding between flavodoxin or flavodoxin coupled to Sepharose was demonstrated. This interaction was ascribed to a structural and charge pairing relationship between flavodoxin and MS which parallels the interaction between P450 reductase and P450 17a-hydroxylase. In both systems, it is the flavodoxin (FMN component) that shows the interaction with the substrate enzyme. It is reasonable to predict that the FMN component of MSR may be the site of interaction with human MS. If so, we suggest that the homology extending across the FMN domains of flavodoxin, P450 reductase and MSR allows for cross-over of interactions with their respective substrates *in vitro* but which would appear, based on the *cblE* disease results, to be unavailable *in vivo*.

Of the eleven mutations identified in this study, three are nonsense mutations. These mutations, R114X in WG2317, 1554del7 in WG1296 and an intronic T→C substitution shared by the two patients, result in stop codons in both alleles of each patient. This suggests that these patients will have very little if any functional MSR protein and should represent the extreme phenotype for MSR deficiency. The first patient, WG2317, presented at 7 weeks with megaloblastic anemia, his older sister having died of the same disease at 9 weeks (15). Severe neurological symptoms followed, including movement disorder, epilepsy and dementia, but he was not diagnosed with MSR deficiency until he was 25 years old, on the basis of high blood homocysteine and low methionine. The second patient, WG1296, also presented early in life, but was not diagnosed with MSR deficiency until 2 years of age. At the time of his diagnosis, he had severe neuropathy and megaloblastic anemia, high blood homocysteine and low methionine (5). Sequence analysis of the intronic T→C substitution suggests that it may strengthen an exonic splicing enhancer (ESE), present in this area of the intron (16). First recognized in 1986, ESEs are found within the sequence of an exon and help to increase the likelihood of a specific splice site being used. We suggest that strengthening of an ESE-like sequence within the intron following nucleotide 903 of the coding sequence allows for the use of an upstream region resembling a 3′ acceptor as an actual splice site, along with a new 5′ donor site recruited 140 bp downstream to produce the insert. The insert creates a frameshift and downstream stop codon when present, preventing the translation of a complete protein. The effect of this mutation is sufficiently strong to make the presence of any normal transcript undetectable by PCR analysis.

The remaining eight mutations involve substitutions or in-frame disruptions of the coding sequence and are found throughout the coding region. Of the seven mutations not identified in the control population, three are located in the vicinity of the proposed FMN binding site. Two more were found to be associated with the FAD and NADPH binding regions. The last two mutations are located in the region connecting the N-terminal FMN binding site to the more C-terminal FAD and NADPH binding sites. This ‘linker’ region has been suggested to have evolved as a result of gene fusion, bringing together the flavodoxin and flavodoxin reductase components of the prokaryotic reductive activation system into a single eukaryotic one, much like the other mammalian members of the FNR family, P450 reductase and nitric oxide synthase (17). The final mutation, L333V, was identified in one of the 50 control alleles used in this study. The fact that this conservative substitution is found in a region of the protein possessing limited sequence homology and was identified in the general population suggests that this mutation is a rare polymorphism. Because no other mutations were evident during the screening process, and because this mutation is infrequent, we cannot rule out that L333V may have a deleterious effect on the patient.

These eight mutations are found in the coding region but should not prevent the production of a complete protein. Most likely, these mutations lead to an enzymatic defect which prevents the reductase from functioning normally. Further experiments, including *in vitro* expression of the mutant proteins, will be required for the biochemical evaluation of their impact on MSR. Together with the previously reported L576del and 1675del4 mutations, 13 mutations in the MSR gene have been discovered (Fig. 1C). The identification of these mutations will allow for diagnostic characterization as well as a possible genotype/phenotype correlation of patients with a deficiency in MSR. Together with a functional expression system and site-directed mutagenesis, the impact of these mutations on the structure and function of the MSR protein may also be determined.

MATERIALS AND METHODS

Cell lines

Ten cell lines from nine families classified as members of the *cblE* complementation group were used in this study. Nine were analyzed in detail. Cell lines WG788, WG1146 (which are from siblings) and WG1836 have been described previously (4). Of the remaining cell lines used: WG1296 was from patient N.J. (5), diagnosed within 2 years of life with microcephaly, significant mental retardation and severe megaloblastic anemia; WG2317 was from the oldest known *cblE* patient, presenting at 7 weeks but diagnosed at 25 years (11); WG1384 was from patient S.M. who died at 5 years of age (5); WG1575 was diagnosed at 6 months of age with microcephaly, developmental delay and megaloblastic anemia;

WG1401 is patient B.S.S. from (5), with megaloblastic anemia, hyperhomocysteinemia and mild methylmalonic aciduria; WG2366 was diagnosed at 11 months of age with megaloblastic anemia, hyperhomocysteinemia and mild developmental delay. Twenty-five additional fibroblast cell lines were used as normal controls.

Materials

Oligonucleotide primers were synthesized by the Sheldon Biotechnology Center (McGill University, Montreal) and ACGT (Toronto, Ontario). AMV reverse transcriptase, *Taq* polymerase and Trizol reagent were purchased from Gibco BRL (Burlington, Ontario). Restriction enzymes were purchased from New England Biolabs (Mississuaga, Ontario) and Gibco BRL. The [α-^{35}S]dATP (12.5 Ci/mol) was purchased from Dupont (Wilmington, DE). The Geneclean III kit was purchased from Bio 101 (Vista, CA), the T/A cloning kit from Invitrogen (Carlsbad, CA) and the Wizard Mini-preps from Promega (Madison, WI). Sequenase kits were obtained from United States Biochemicals (Oakville, Ontario).

DNA/RNA isolation and reverse transcription of total mRNA

To isolate genomic DNA, cell pellets were re-suspended in 300 µl digestion buffer (100 mMNacl, 10 mM Tris–HCl pH 8, 25 mM EDTA pH 8 and 0.5% SDS). Proteinase K was added to a final concentration of 0.1 mg/ml. Samples were incubated at 50°C overnight before phenol–chloroform extraction and precipitation using 0.5 vol 7.5 M ammonium acetate and 2 vol 100% cold ethanol. Precipitated DNA was re-suspended in 200 µl TE at pH 7.6. Total cellular RNA was isolated from fibroblast pellets by a previously published method (18) Reverse transcription was carried out using 25 µg total RNA in reactions containing 2.5 U AMV reverse transcriptase and 500 ng MSR-specific oligonucleotide primer (2101C in ref. 4) in a total reaction volume of 54 µl. Resultant cDNA was used as template for PCR.

Heteroduplex and SSCP analyses and DNA sequencing

Heteroduplex and SSCP analyses were carried out as described previously (4,12). For heteroduplex analysis, PCRs for nine overlapping cDNA segments (Table 1) were performed in reactions containing 3 µl template, 1 µl each dNTPs (10 mM) and 3 U *Taq* polymerase in a 46 µl volume. For SSCP analysis, PCRs were performed using 1 µl each of dTTP, dGTP and dCTP (0.625 mM), 0.5 µl dATP (0.625 mM) and 1 µl [α-^{35}S]dATP (12.5 Ci/mol). PCR products were verified by agarose gel electrophoresis before undergoing heteroduplex or SSCP analysis. Fragments displaying shifts by either heteroduplex or SSCP analysis were subcloned and sequenced, or sequenced directly. Sequencing was performed using the primers of the initial PCR amplification on both genomic DNA and cDNA. Mutations were identified through sequencing of cDNA, genomic DNA or both.

Confirmation of mutations

The confirmation of mutations in genomic DNA was made by PCR-dependent diagnostic tests (Table 2). These tests assayed for the creation or destruction of a particular restriction site as the result of a specific mutation. If the mutation did not change a restriction site, one was created through the use of an oligonucleotide in which one or more of the 3′ end nucleotides was altered, as shown in Table 2. This altered oligonucleotide generated a restriction site when found in combination with the specific mutation, cutting the appropriate PCR product to generate the expected fragments. Genomic PCR was carried out using 2 µl template, 500 ng specific primers, 1 µl each dNTPs (10 mM), and 3 U *Taq* polymerase in a 46 µl volume. Digestion was carried out using 10–15 µl of the PCR product with the appropriate restriction enzyme and buffers for 4 h at the appropriate temperatures before subjecting the samples to electrophoresis on an 8% acrylamide gel. The oligonucleotides used to generate PCR products specific to diagnostic tests and their sequences are shown in Table 2.

PCR determination of transcripts in WG1296 and WG2317

To determine the presence of normal transcript in WG1296 and WG2317, a PCR was performed using oligonucleotides AY5 5′-GGTAGAATTGGACATTTCAAATAC and AY6 5′-TATGTTTGGCTGAAGAACTGAAG. The PCR was carried out using 3 µl template, 500 ng specific primers, 1 µl each dNTPs (10 mM) and 3 U *Taq* polymerase in a 50 µl volume. A second PCR, using primers AB581 and 1103A was performed at the same time and using similar conditions to ensure that the template was not degraded.

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REFERENCES

1. Rosenblatt, D.S. (1995) Inherited disorders of folate transport and metabolism. In Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D. (eds), *The Metabolic and Molecular Bases of Inherited Disease*. 7th edn, McGraw-Hill, New York, NY, pp. 3111–3128.
2. Fenton, W.A., and Rosenberg, L.E. (1995) Inherited disorders of cobalamin transport and metabolism. In Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D. (eds), *The Metabolic and Molecular Bases of Inherited Disease*. 7th edn, McGraw-Hill, New York, NY, pp. 3129–3149.
3. Banerjee, R. (1997) The Yin–Yang of cobalamin biochemistry. *Chem. Biol.*, **4**, 175–186.
4. Leclerc, D., Wilson, A., Dumas, R., Gafuik, C., Song, D., Watkins, D., Heng, H.H., Rommens, J.M., Scherer, S.W., Rosenblatt, D.S. and Gravel, R.A. (1998) Cloning and mapping of a cDNA for methionine synthase reductase, a flavoprotein defective in patients with homocystinuria. *Proc. Natl Acad. Sci. USA*, **95**, 3059–3064.
5. Watkins, D. and Rosenblatt, D.S. (1989) Functional methionine synthase deficiency (cblE and cblG): clinical and biochemical heterogeneity. *Am. J. Med. Genet.*, **34**, 427–434.
6. Harding, C.O., Arnold, G., Barness, L.A., Wolff, J.A. and Rosenblatt, D.S. (1997) Functional methionine synthase deficiency due to *cblG* disorder: a report of two patients and a review. *Am. J. Med. Genet.*, **71**, 384–390.
7. Watkins, D. and Rosenblatt, D.S. (1988) Genetic heterogeneity among patients with methylcobalamin deficiency. Definition of two complementation groups, *cblE* and *cblG*. *J. Clin. Invest.*, **81**, 1690–1694.
8. Schuh, S., Rosenblatt, D.S., Cooper, B.A., Schroeder, M.L., Bishop, A.J., Seargeant, L.E. and Haworth, J.C. (1984) Homocystinuria and

megaloblastic anemia responsive to vitamin B12 therapy. An inborn error of metabolism due to a defect in cobalamin metabolism. *N. Engl. J. Med.*, **310**, 686–690.
9. Rosenblatt, D.S., Cooper, B.A., Pottier, A., Lue-Shing, H., Matiaszuk, N. and Grauer, K. (1984) Altered vitamin B12 metabolism in fibroblasts from a patient with megaloblastic anemia and homocystinuria due to a new defect in methionine biosynthesis. *J. Clin. Invest.*, **74**, 2149–2156.
10. Gulati, S., Chen, Z., Brody, L.C., Rosenblatt, D.S. and Banerjee, R. (1997) Defects in auxiliary redox proteins lead to functional methionine synthase deficiency. *J. Biol. Chem.*, **272**, 19171–19175.
11. Chen, Z. and Banerjee, R. (1998) Purification of soluble cytochrome b_5 as a component of the reductive activation of porcine methionine synthase. *J. Biol. Chem.*, **273**, 26248–26255.
12. Wilson, A., Leclerc, D., Saberi, F., Campeau, E., Hwang, H.Y., Shane, B., Phillips, J.A.III, Rosenblatt, D.S. and Gravel, R.A. (1998) Functionally null mutations in patients with the *cblG* variant form of methionine synthase deficiency. *Am. J. Hum. Genet.*, **63**, 409–414.
13. Hegesh, E., Hegesh, J. and Kaftory, A. (1986) Congenital methemoglobinemia with a deficiency of cytochrome b_5. *N. Engl. J. Med.*, **314**, 757–761.
14. Jenkins, C.M. and Waterman, M.R. (1994) Flavodoxin and NADPH-flavodoxin reductase from *Escherichia coli* support bovine cytochrome P450 17Hydroxylase activities. *J. Biol. Chem.*, **269**, 27401–27408.
15. Steen, C., Rosenblatt, D.S., Scheying, H., Braeuer, H.C. and Kohlschutter, A. (1997) Cobalamin E (cblE) disease: a severe neurological disorder with megaloblastic anemia, homocystinuria and low serum methionine. *J. Inher. Metab. Dis.*, **20**, 705–706.
16. Cooper, T.A. and Mattox, W. (1997) Gene regulation '97: The regulation of splice-site selection, and its role in human disease. *Am. J. Hum. Genet.*, **61**, 259–266.
17. Shen, A.L., Porter, T.D., Wilson, T.E. and Kasper, C.B. (1989) Structural analysis of the FMN binding domain of NADPH-cytochrome P-450 oxidoreductase by site-directed mutagenesis. *J. Biol. Chem.*, **264**, 7584–7589.
18. Chirgwin, J.M., Przybyla, A.E., Macdonald, R.J. and Rutter, W.J. (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry*, **18**, 5294–5299.