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Functionally Null Mutations in Patients with the *cblG*-Variant Form of Methionine Synthase Deficiency

A. Wilson,[1,3] D. Leclerc,[1] F. Saberi,[1,3] E. Campeau,[1,3] H. Y. Hwang,[6] B. Shane,[6] J. A. Phillips III,[7] D. S. Rosenblatt,[2,3,4,5] and R. A. Gravel[1,3,4]

[1]Medical Research Council Group in Medical Genetics, Montreal Children's Hospital, [2]Royal Victoria Hospital, and [3]Research Institutes and Department of Biology, Departments of [4]Human Genetics and Pediatrics, and [5]Department of Medicine, McGill University, Montreal; [6]Department of Nutritional Sciences, University of California, Berkeley; and [7]Vanderbilt University School of Medicine, Nashville

Summary

Methionine synthase (MS) catalyses the methylation of homocysteine to methionine and requires the vitamin B_{12} derivative, methylcobalamin, as cofactor. We and others have recently cloned cDNAs for MS and described mutations associated with the *cblG* complementation group that correspond to MS deficiency. A subset of *cblG*, known as "*cblG* variant," shows no detectable MS activity and failure of [^{57}Co]CN cobalamin to incorporate into MS in patient fibroblasts. We report the mutations responsible for three *cblG*-variant patients, two of them siblings, who presented with neonatal seizures, severe developmental delay, and elevated plasma homocysteine. Cell lines from all three patients were negative by northern blotting, though trace MS mRNA could be detected by means of phosphorimage analysis. Reverse transcriptase–PCR, SSCP, and nucleotide sequence analysis revealed four mutations. All were functionally null, creating either a frameshift with a downstream stop codon or an insert containing an internal stop codon. Of the two mutations found in the siblings, one of them, intervening sequence (IVS)-166A→G, generates a cryptic donor splice site at position −166 of an intron beginning after Leu113, resulting in a 165-bp insertion of intronic sequence at junction 339/340. The second is a 2-bp deletion, 2112delTC. Mutations in the third patient include a G→A substitution, well within the intron after Lys203, which results in intronic inserts of 128 or 78 bp in the mRNA. The second mutation is a 1-bp insertion, 3378insA. We conclude that the absence of MS protein in these *cblG* variants is due to mutations causing premature translation termination and consequent mRNA instability.

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Address for correspondence and reprints: Dr. R. A. Gravel, MUHC–Montreal Children's Hospital Research Institute, 4060 Ste-Catherine Street West, Montreal, Quebec H3Z 2Z3,Canada. E-mail: mc84@musica.mcgill.ca

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Introduction

Methionine synthase (MS) is a cobalamin-dependent enzyme that catalyzes the methylation of homocysteine to methionine, using 5-methyltetrahydrofolate as a methyl donor (Fenton and Rosenberg 1995; Rosenblatt 1995). The cobalamin cofactor acts as an intermediate methyl carrier, such that the methyl group of 5-methyltetrahydrofolate is transferred first to the enzyme-bound cob(I)alamin, to form methyl cobalamin, with subsequent transfer to homocysteine and regeneration of the cob(I)alamin cofactor (Banerjee 1997). Over time, the highly reactive cob(I)alamin cofactor may become oxidized to cob(II)alamin, rendering the enzyme inactive. Regeneration of the functional enzyme takes place through the reductive methylation of the cob(II)alamin, occurring through a distinct enzyme and using S-adenosyl methionine as the source of the methyl group (Ludwig and Matthews 1997). Recently, cDNAs coding for MS (Gulati et al. 1996; Leclerc et al. 1996; Li et al. 1996; Chen et al. 1997) as well as for the MS reductase responsible for its reactivation (Leclerc et al. 1998) were cloned, making mutation detection and characterization of patient cell lines possible.

Deficiency of MS activity has been shown to result in the biochemical phenotypes of hyperhomocysteinemia, homocystinuria, hypomethioninemia, and megaloblastic anemia without methylmalonic aciduria (Watkins and Rosenblatt 1989; Harding et al. 1997). Patients show a range of clinical symptoms, including severe developmental delay, ataxia, cerebral atrophy, neonatal seizures, and blindness. Most *cblG* patients have reduced MS activity, with normal accumulations of labeled [^{57}Co]CN cobalamin associated with the defective MS enzyme in fibroblast-loading experiments (Sillaots et al. 1992). However, three patient cell lines, two of which are from siblings, show a reduced accumulation of labeled cobalamin, none of which is bound to MS. The MS activity of these cells is almost completely undetectable (Sillaots et al. 1992; Gulati et al. 1997). Because these three cell lines differ from the majority of *cblG* lines, they have been classified as "*cblG* variants" (Sillaots et al. 1992).

All three patients experienced severe developmental delay, with neonatal seizures and elevated plasma homocysteine.

In this study, we report four distinct mutations in the *cblG*-variant patients. All four mutations would be expected to result in the premature truncation of a complete MS protein. However, these mutations lead to mRNA instability and insufficient mRNA to produce any significant amount of MS enzyme. These results show that the molecular defects in *cblG* variants are consistently associated with a biochemically null phenotype.

Material and Methods

Cell Lines

Skin fibroblasts from the *cblG*-variant cell lines WG1670, WG1671, and WG1655 were obtained from the Montreal Children's Hospital Cell Repository for Mutant Human Cell Strains. DNA from the mother of siblings WG1670 and WG1671 as well as from the siblings themselves was kindly provided by J. P. Pfotenhauer (Vanderbilt University School of Medicine, Nashville).

WG1671, the first child of unrelated parents, developed generalized seizures at 3 d of age. He became progressively hypotonic, and he suffered respiratory failure at 10 wk of age. Elevated plasma and urine homocysteine (23 and 490 μmol/liter, respectively) but low plasma methionine (5 μmol/liter) without macrocytic anemia led to the initial diagnosis of methylenetetrahydrofolate reductase (MTHFR) deficiency. He was treated with folinic acid, vitamin B_{12}, vitamin B_6, betaine, and methionine and was well enough to be weaned from the respirator by 14 wk of age. By 2 years of age, he had severe psychomotor retardation and microcephaly. A definitive diagnosis of MS deficiency was made by means of complementation analysis of cultured fibroblasts, which placed him in the *cblG* complementation group. He was subsequently treated with vitamin B_{12}, betaine, and aspirin, and, at age 8 years, methionine was added. He has required femoral osteotomies and bilateral adductor- and heel-cord release for neuromuscular hip dislocations and contractures. At age 10 years, he has short stature, microcephaly, rotatory nystagmus, thin fingers, and spasticity. He smiles but is not able to sit or speak.

WG1670, the younger sister of the patient described above, was found to have elevated plasma homocysteine (52 μmol/liter) and low methionine (8 μmol/liter) at 6 d of age. She was presumed to have MTHFR deficiency and was started on folinic acid, vitamin B_{12}, vitamin B_6, betaine, and methionine, but these were discontinued by her mother after a few days because the child appeared to be doing well. At 3 mo of age, she had seizures and respiratory distress, and medications were restarted. By 18 mo of age, she was microcephalic and severely developmentally delayed. At 2 years of age, the diagnosis of *cblG* was established, and she was treated with vitamin B_{12}, betaine, and aspirin; at age 6 years, methionine supplementation was added. At 9 years, she has short stature, microcephaly, rotatory nystagmus, and pes planus. She is able to walk, responds to simple commands, and can speak a few words.

WG1655 presented with short stature, failure to thrive, progressive weakness, hypotonia, ocular nystagmus, jaundice, feeding difficulties, and diarrhea at 7–10 wk of age (Wilden and Scott 1992). He had severe megaloblastic anemia and neutropenia, homocysteinemia, hypomethioninemia, and formiminoglutamic aciduria without methylmalonic aciduria, which led to the diagnosis of a defect in methionine synthesis. Treatment with hydroxycobalamin, methionine, and folinic acid resulted in improved metabolite levels, improvement of tone, and reduction of nystagmus, but poor growth, developmental delay, feeding difficulties requiring a gastrostomy, persistent anemia, and immunological deficits at age 4 years (Wilden and Scott 1992).

All three of the patients—WG1670, WG1671, and WG1655—were classified as *cblG* variants, in accordance with findings from tests as described by Sillaots et al. (1992). Test results showed reduced accumulations of labeled cobalamin, virtually none of which associated with the MS enzyme, and almost undetectable MS activity.

Materials

Oligonucleotide primers were synthesized by R. Clarizio (Montreal Children's Hospital Research Institute Oligonucleotide Synthesis Facility, Sheldon Biotechnology Center, McGill University, and ACGT Corporation, Toronto). AMV reverse transcriptase (RT), *Taq* polymerase, Trizol, and DNAzol reagent were purchased from Gibco BRL. Restriction enzymes were purchased from New England Biolabs. The α-[^{35}S] dATP (12.5 Ci/mol) was purchased from Dupont, and the α-[^{32}P] dATP was from ICN Pharmaceuticals. The random-primed DNA-labeling kit was from Boehringer. The Geneclean III kit was purchased from Bio 101, the T/A cloning kit from Invitrogen, and the Wizard Mini-preps from Promega. Sequenase kits were obtained from United States Biochemicals.

Northern Blot Analysis

Total RNA from human fibroblast cell lines WG1670, WG1671, and WG1655 and control 8074 was isolated by use of the Trizol reagent. Cells were lysed in culture dishes as recommended by the manufacturer. RNA con-

centrations were measured spectrophotometrically at 260 nm. A total of 15 μg of RNA from each patient was denatured and subjected to electrophoresis in 1.5% agarose formaldehyde gels and was then transferred to Zetabind nylon membranes. The membranes were prehybridized at 65°C in 0.5 M phosphate buffer (pH 7.0), 1 mM EDTA, 7% SDS, 1% BSA, and 50 μg single-stranded salmon-sperm DNA for 2 h. Hybridization was performed overnight in fresh prehybridization buffer with a ^{32}P-labeled human MS cDNA probe. The cDNA probes, generated through PCR with oligonucleotides D1730 and D1733 (Leclerc et al. 1996), were labeled with α-[^{32}P] dATP by use of the random-primed DNA-labeling kit. A phosphorimage analyzer (Fuji BAS 2000 Bio-Imaging Analyser; Fuji Medical Systems) was used to detect radioactive signals.

Mutation Analysis

Total cellular RNA was isolated from fibroblast pellets by use of the method of Chirgwin et al. (1979). Genomic DNA was isolated with the use of DNAzol reagent according to the manufacturer's specifications. Reverse transcription was performed with 25 μg total RNA in reactions containing 2.5 U of AMV RT and 500 ng of MS-specific terminal oligonucleotide primer (1107A in the report by Leclerc et al. [1996]) in a total reaction volume of 54 μl. Resultant cDNA was used as template for PCR. PCR for five overlapping cDNA segments (table 1) was performed in reactions containing 5 ml of template; 1 μl each of dTTP, dGTP, and dCTP (10 mM); 0.5 ml of dATP (10 mM); and 1 μl of α-[^{35}S] dATP (12.5 Ci/mol). SSCP analysis was performed as described elsewhere (Leclerc et al. 1996). Fragments displaying shifts were either subcloned and sequenced or sequenced directly. Sequencing was performed with use of the primers of the initial PCR amplification, on both genomic DNA and cDNA.

The confirmation of mutations in genomic DNA was made by PCR-dependent diagnostic tests. Genomic PCR was performed with 3 μl of template, 500 ng of specific primers, 1 μl each of dNTPs (10 mM), and 3 U of *Taq* polymerase in a 46-μl volume. Mutation 2112delTC was identified through genomic sequencing and was confirmed by heteroduplex analysis by use of oligonucleotides AA111 (5′-GTG ATA GGA AGC CAG ATT GAG) and 511B (5′-GAA AAT TTC CCC ATA TTT TGC TAA C) as primers for the PCR reaction. The product was heated to 95°C for 3 min, cooled to room temperature, and run on 8% polyacrylamide gels. Mutation IVS-166A→G was identified through genomic sequencing using primer pairs 1939 (5′-CTA CCT CAT GGT TTG GGA GGA GAA) and 1938 (5′-GAT TCA TGA CCA TCT AAT ACT CAG). The mutation was detected by a test for the loss of an artificially created *Mse*I restriction site. Primer pairs used for this diagnostic test were 2802A (5′-CTT GTC TTT CCT TGC GCC TTT TA) and 2808C (5′-AGA GCA CAT GTT CAT CCG TAG GC), where the underlined T in 2802A replaced the C in the normal sequence. To test for the mutation, 15 μl of PCR product was digested in 2.2 μl of New England Biolab's Buffer 2, 2.2 μl of 10 × BSA, and 2.5 μl of *Mse*I. The G→A substitution found in the intron beginning after Lys203 was identified through genomic sequencing by use of primer pairs 309A (5′-GAG CAG GCC AAA GGA CTT CTG GAT) and 2802B (5′-CTG CTC TTC AAC TGA GGA GTG GCT). The mutation was confirmed by a test for the presence of an artificially created *Bfa*I site. The PCR primers used were AE358 (5′-TTT ACA GAT TCT ATT TTT TTG TTC T) and AE359 (5′-TCT GAT TCC CAC AAC AAT GAA A), where the underlined C in AE358 replaced the G in the normal sequence. To test for the mutation, 15 μl of PCR product was digested in 1.9 μl of NE Buffer 4 and 2 μl of *Bfa*I. Mutation 3378insA was first identified through genomic sequencing with primer pairs 1774 (5′-TGC CTC TCA GAC TTC ATC GCT CCC) and AE373 (5′-

Table 1

cDNA Sequence Segments Used in SSCP Analysis

PCR Segment Number	Nucleotide Position[a]	Annealing Temperature (°C)	Primer Pairs[b]	Uncut PCR Product (bp)[c]	Enzyme	Digestion Products (bp)[d]
1	−56–635	66	3107/1209A	690	*Pst*I	136, 286, 268
2	532–1657	62	309A/1827	1,125	*Alu*I	261, 216, 363, 105, 180
3	1468–2057	60	1907A/1907B	590	*Hin*fI	485, 105
4	1963–2698	60	1406E/1773	735	*Ava*II	165, 176, 394
5	2610–3856	60	1783/1107A	1,246	*Mse*I	184, 196, 203, 414, 249

[a] Counted from the A of the ATG initiation codon.
[b] Sense/antisense oligonucleotides used to generate each segment.
[c] Expected size.
[d] Size of PCR fragments after digestion, listed in order from 5′ end of segment.

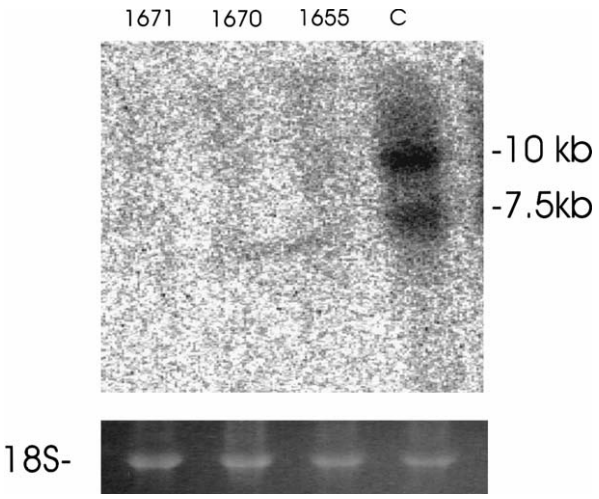

Figure 1 Results of northern analysis, showing levels of mRNA in variant cell lines WG1670, WG1671, and WG1655. The control pattern is on the right, with bands at 10 and 7.5 kb compared with molecular-weight standards. At the bottom is the pattern obtained for 18S RNA as a control for differences in sample loading.

CTC CCT ACT GCC TGG CCT CTG TC). The diagnostic test for the 3378insA mutation was performed by assaying for the loss of an artificially created *Afl*II site. The oligonucleotide primers used for PCR were AF345 (5′-GAC TAC AGC AGC ATC ATG CTT AA) and AH450 (5′-GAC ACT GGT TCT AAG GGC TGA T), where the underlined C and T in AF345 replaced the normal nucleotides G and C, respectively. To test for the mutation, 15 μl of PCR product was digested in 2 μl of New England Biolab's Buffer 2, 2 μl of 10 × BSA, and 1 μl of *Afl*II. All digestions were performed at 37°C overnight before the samples were subjected to electrophoresis on 8% polyacrylamide gels.

Results

Fibroblast cell lines WG1670, WG1671, and WG1655 were analyzed by means of northern blot, with a 1-kb PCR-generated fragment from the human MS cDNA used as a probe. Normal cells showed bands at 7.5 and 10 kb, as expected (Chen et al. 1997). In contrast, none of the patient samples gave a detectable signal under the same exposure conditions, although trace signals could be detected, after long exposure, by phosphorimage analysis (fig. 1). It was possible to amplify these trace-level transcripts through RT-PCR, which allowed an analysis of the resultant cDNA. The cDNA was divided into five overlapping segments, as shown in table 1. These fragments were amplified and evaluated for possible mutations.

When RT-PCR products were run with use of agarose gel electrophoresis, slower-running bands were found associated with fragment 1 of both WG1671 and WG1655 (fig. 2*A*). Subcloning and sequencing of the larger band associated with WG1671 revealed a 165-bp insertion of intronic DNA at junction 339/340 after exon 3 (numbered from the A of the initiation codon; fig. 2*B*). Sequencing of genomic DNA showed the insertion to be the result of a substitution, IVS-166A→G, which generates a cryptic 3′ acceptor splice site at position −166 of the intron beginning after Leu113. The insertion contains an in-frame stop codon 9 bp into the sequence, an indication that translation is terminated. The mutation was confirmed by diagnostic PCR with use of an artificially created *Mse*I restriction site (fig. 3*A*). Alteration of the sense oligonucleotide created an *Mse*I cut site in control DNA. The presence of the A→G mutation eliminated the restriction site. The mutation was confirmed as heterozygous in genomic DNA from both siblings (WG1670 and WG1671), since both cut and uncut bands were present on the diagnostic gel. DNA from the mother was also made available, and testing for the mutation gave negative results.

The second mutation in WG1670 and WG1671 was detected as a heteroduplex during SSCP analysis of frag-

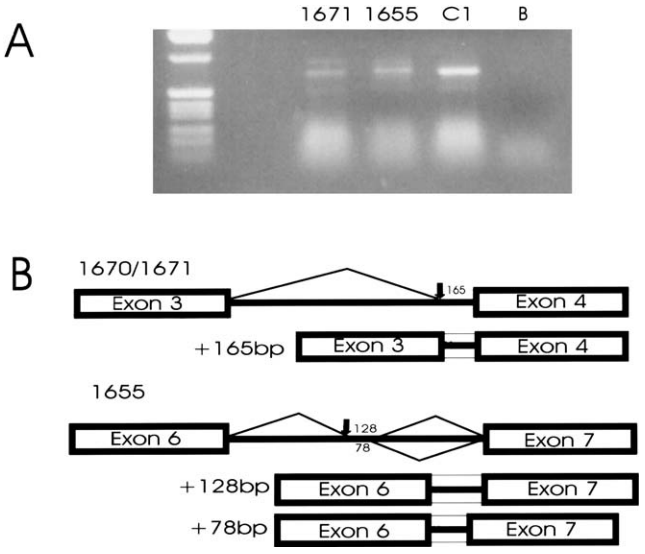

Figure 2 mRNA abnormalities in mutant fibroblast lines. *A*, Agarose gel of RT-PCR products showing extra bands indicative of splice defects in fibroblast lines WG1671 and WG1655. C1 is a control cell line; B, the water blank. The bands on the left are molecular-weight standards. *B*, Mechanism of splicing defects. In cell lines WG1670 and WG1671, the A→G substitution at position −166 of the intron after exon 3, beginning at Leu113, creates a 3′ acceptor site 165 bp upstream of the normal acceptor site. This new 3′ site replaces the original downstream site, which has not been altered. In cell line 1655, the G→A substitution near the middle of the intron after exon 6, beginning at Lys203, creates a 3′ acceptor site and consequent generation of cryptic donor sites 78 and 128 bp farther downstream. All inserts contain stop codons.

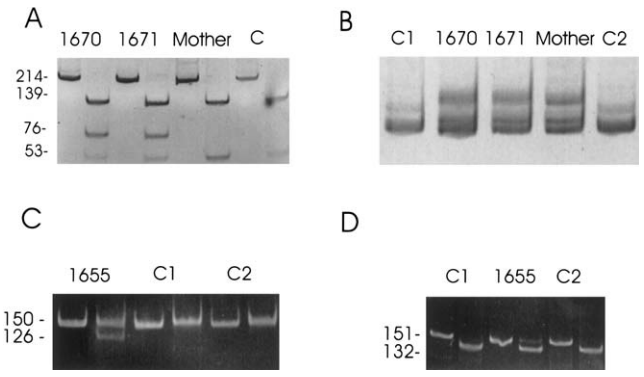

Figure 3 DNA-based diagnostic tests for the identified mutations. DNA was amplified by PCR and either digested or not digested with the designated restriction enzyme, and the fragments were resolved by PAGE. *A*, Test for IVS-166A→G. An artificially created *Mse*I site was introduced through alteration of the sense oligonucleotide 2802A, so that *Mse*I digestion of control DNA cleaves the 214-bp PCR product into fragments of 139, 53, and 23 bp (the last not shown). The mutant sequence gives 139- and 76-bp fragments as cleavage products. *B*, Test for 2112delTC. Electrophoresis of the PCR products leaves trailing heteroduplexes in samples where the mutation is heterozygous. *C*, Test for G→A mutation in intron after Lys203. An artificially created *Bfa*I site was introduced through alteration of the sense oligonucleotide AE358, so that *Bfa*I digestion results in cleavage of the 150-bp PCR product to 126 and 24 bp (latter not shown) if the mutation is present. *D*, Test for 3378insA. An artificially created *Afl*II was introduced through alteration of the sense oligonucleotide AF345, so that digestion with *Afl*II cleaves the 151-bp PCR product to 132 and 19 bp (latter not shown) if the wild-type sequence is present. For all panels, C, C1 and C2 are control DNA samples, and the numbered samples correspond to the WG designation of patient cell lines.

ment 4. It was sequenced directly from cDNA and genomic DNA PCR products and was shown to be due to a 2-bp deletion, 2112delTC, resulting in a frameshift and a downstream stop codon. A diagnostic test for the mutation was made by heteroduplex analysis of PCR products amplified from genomic DNA (fig. 3*B*). It was confirmed in both siblings. DNA from the mother was negative for the IVS-166A→G mutation and positive for 2112delTC, which confirmed that the mutations are on separate chromosomes (fig. 3*A* and *B*).

Sequencing of the slower-running bands associated with the RT-PCR of fragment 1 from cell line WG1655 yielded two insertions in the mRNA, one of 78 bp and another of 128 bp, beginning after Lys203. The 78-bp insertion was found to be a truncated version of the 128-bp insertion, missing the last 50 bp. By means of genomic sequencing, the insertions were discovered to be the result of a G→A substitution, near the center of the intron after exon 6, that created a single cryptic 3′ acceptor splice site (fig. 2*B*). For each insert, a cryptic 5′ donor splice site was recruited to complete the new exon, one at 78 bp and the other at 128 bp downstream of the mutation. Neither of the newly created 5′ donor sites is preferred, since the two transcripts appear to occur in equal amounts. An in-frame stop codon occurs 9 bp into the insertions. The mutation was confirmed by use of diagnostic PCR on genomic DNA, with an artificially created *Bfa*I restriction site (fig. 3*C*). Alteration of the sense oligonucleotide created a *Bfa*I restriction site if the G→A mutation was present. Because the mutation was heterozygous, both cut and uncut bands were present on the diagnostic gel.

In RT-PCR and SSCP analysis of segment 5, the second mutation in WG1655 was detected as a band shift, relative to the controls. This segment was subcloned and sequenced from both cDNA and genomic DNA. A 1-bp insertion, 3378insA, was detected, which results in a frameshift and a downstream stop codon. The mutation was confirmed by diagnostic PCR on genomic DNA, with use of an artificially created *Afl*II restriction site. Alteration of the sense oligonucleotide created an *Afl*II site that was eliminated if the inserted A was present (fig. 3*D*). Because the mutation was heterozygous, both cut and uncut bands were present in the diagnostic gel. Screening for the four mutations in the other *cblG* cell lines gave negative results, showing that the mutations are unique to these individuals.

Discussion

All four mutations described here—the two splice mutations and two frameshifts—are compatible with premature termination of translation and consequent instability of MS mRNA, as confirmed by the near-complete absence of mRNA detected by northern blotting. These molecular defects provide an explanation for the absence of cobalamin-associated MS and for the profound deficiency of enzyme activity in cultured fibroblasts that defines the *cblG*-variant group (Sillaots et al. 1992). In contrast, other *cblG* patients incorporate [^{57}Co]CN cobalamin into their MS protein, although they too have deficient MS activity.

The three *cblG*-variant patients showed onset of disease in the first few days or weeks of life, with profound neurological findings, hyperhomocysteinemia, and hypomethioninemia. Poor growth, hypotonia, nystagmus, and developmental delay were common features. One of the three patients (WG1655) had severe megaloblastic anemia. These findings are similar to those for patients with severe *cblG* disease, as described by Watkins and Rosenblatt (1989). Although the onset of disease may also be early in non–*cblG*-variant patients, it may not occur until either later in childhood or even adulthood (Carmel et al. 1988). Patients with *cblE* disease, due to a deficiency in MS reductase (Leclerc et al. 1998), also show a broad spectrum of clinical findings that are similar to those seen in the *cblG* patients.

It is significant that all four alleles in the two families

we studied had null-type mutations. Thus, the relatively rare *cblG* variants do not share a common genetic abnormality but instead share a biochemical characteristic, the absence of MS protein. Although this leads to a severe early-onset *cblG* phenotype, our findings confirm that complete MS deficiency is not lethal.

Acknowledgments

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