

## The cblD Defect Causes Either Isolated or Combined Deficiency of Methylcobalamin and Adenosylcobalamin Synthesis\*

Received for publication, July 9, 2004, and in revised form, July 26, 2004  
Published, JBC Papers in Press, August 2, 2004, DOI 10.1074/jbc.M407733200

Terttu Suormala‡, Matthias R. Baumgartner‡§, David Coelho‡, Petra Zavadakova¶, Viktor Kožich¶, Hans Georg Koch||, Martin Berghäuser||, James E. Wraith\*\*, Alberto Burlina‡‡, Adrian Sewell§§, Jürgen Herwig§§, and Brian Fowler‡¶¶

From the ‡Metabolic Unit, University Children's Hospital, Römergasse 8, CH-4058 Basel, Switzerland, the ¶Institute of Inherited Metabolic Diseases, Charles University, 1st Faculty of Medicine, 12808 Praha 2, Czech Republic, the ||Department of Pediatrics, University Hospital, Münster D48149, Germany, the \*\*Willink Biochemical Genetics Unit, Royal Manchester Children's Hospital, Manchester M27 4HA, United Kingdom, the ‡‡Department of Pediatrics, University of Padua 35128, Italy, and the §§Department of Pediatrics, Johann Wolfgang Goethe-University, Frankfurt am Main D-60590, Germany

Intracellular cobalamin is converted to adenosylcobalamin, coenzyme for methylmalonyl-CoA mutase and to methylcobalamin, coenzyme for methionine synthase, in an incompletely understood sequence of reactions. Genetic defects of these steps are defined as cbl complementation groups of which cblC, cblD (described in only two siblings), and cblF are associated with combined homocystinuria and methylmalonic aciduria. Here we describe three unrelated patients belonging to the cblD complementation group but with distinct biochemical phenotypes different from that described in the original cblD siblings. Two patients presented with isolated homocystinuria and reduced formation of methionine and methylcobalamin in cultured fibroblasts, defined as cblD-variant 1, and one patient with isolated methylmalonic aciduria and deficient adenosylcobalamin synthesis in fibroblasts, defined as cblD-variant 2. Cell lines from the cblD-variant 1 patients clearly complemented reference lines with the same biochemical phenotype, *i.e.* cblE and cblG, and the cblD-variant 2 cell line complemented cells from the mutant classes with isolated deficiency of adenosylcobalamin synthesis, *i.e.* cblA and cblB. Also, no pathogenic sequence changes in the coding regions of genes associated with the respective biochemical phenotypes were found. These findings indicate heterogeneity within the previously defined cblD mutant class and point to further complexity of intracellular cobalamin metabolism.

Cobalamin (cbl,<sup>1</sup> vitamin B<sub>12</sub>) serves as the cofactor for two enzymes in humans. Mitochondrial methylmalonyl-CoA mu-

tase (MMA-CoA mutase; EC 5.4.99.2) requires 5-deoxyadenosylcobalamin (AdoCbl), and N<sup>5</sup>-methyltetrahydrofolate:homocysteine methyltransferase (methionine synthase, EC 2.1.1.13) is dependent on methylcobalamin (MeCbl).

To fulfill its cofactor function dietary cbl must be absorbed, transported in the bloodstream, and taken up into cells in a complex series of processes involving specific receptors and carrier proteins (1). Intracellular processing is also complex and not fully understood. After release of lysosomal cbl into the cytosol, reduction of cob(III)alamin to cob(I)alamin is followed by reductive methylation on the methionine synthase enzyme in the cytosol, or by attachment of an adenosyl group in the mitochondrion. A number of rare defects in these intracellular processing steps are known, causing disease of varying severity. So far eight cbl complementation groups have been linked to these disorders (see "Discussion" for further details).

The cblE (MIM 236270) and cblG (MIM 250940) complementation groups represent defects of methionine synthase reductase (EC 2.1.1.135; *MTRR* gene) and methionine synthase (*MTR* gene), respectively, both causing isolated homocystinuria (2–4). Banerjee and coworkers have shown that another system, comprising soluble cytochrome b<sub>5</sub> and novel reductase 1 (NR1), is able to reductively activate methionine synthase, in addition to methionine synthase reductase. However, the functional significance of this system remains unclear (5).

The cblA (MIM 251100), cblB (MIM 251110), and cblH (MIM 606169) complementation groups are linked to processes unique to AdoCbl synthesis and cause isolated methylmalonic aciduria (MMA-uria) (6, 7). Genes for the cblA (*MMAA*) and cblB (*MMAB*) groups have recently been described, although the corresponding proteins have not yet been characterized. The deduced amino acid sequence of the *MMAA* gene points to a transporter or accessory protein involved in translocation of cbl into the mitochondrion (8). The *MMAB* gene appears to code for cbl adenosyltransferase (EC 2.5.1.7) (9). Only one patient has been designated to the cblH complementation group, and nothing is known about the corresponding gene or protein (7). Isolated MMA-uria is also caused by defects of the MMA-CoA mutase apoenzyme (EC 5.4.99.2), designated the mut complementation group. The majority of patients have complete enzyme deficiency (mut<sup>0</sup>), whereas some (mut<sup>-</sup>) show residual enzyme activity with reduced affinity for AdoCbl, and hydroxocobalamin (OH-Cbl) responsiveness in fibroblasts (6).

The cblF (MIM 277380), cblC (MIM 277400), and cblD (MIM 277410) complementation groups affect early steps of intracellular cbl processing and cause combined homocystinuria with MMA-uria (1). The cblF defect is due to disturbed lysosomal

\* This work was supported by the Swiss National Science Foundation (Grant 3200-066878), and in part by the research project VZ111100003 of Charles University, 1st Faculty of Medicine, Prague, Czech Republic. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Present address: Division of Metabolism and Molecular Pediatrics, University Children's Hospital, CH-8032 Zürich, Switzerland.

¶¶ To whom correspondence should be addressed. Tel: 41-(0)61-685-6275; Fax: 41-(0)61-685-6566; E-mail: Brian.Fowler@ukbb.ch.

<sup>1</sup> The abbreviations used are: cbl, cobalamin; AdoCbl, 5-deoxyadenosylcobalamin or adenosylcobalamin; CN-Cbl, cyanocobalamin; MeCbl, methylcobalamin; OH-Cbl, hydroxocobalamin; MMA, methylmalonyl or methylmalonic acid; MTHFR, methylenetetrahydrofolate reductase; *MTR*, gene coding for methionine synthase; *MTRR*, gene coding for methionine synthase reductase; NR1, novel reductase 1; MRI, magnetic resonance imaging; SNP, single nucleotide polymorphism.

release of OH-Cbl into cytoplasm (10). The defect in the *cblC* and *cblD* groups is likely to involve cytosolic reduction of *cbl* as suggested by two reports (11, 12), but the exact metabolic steps and mechanisms remain unclear. Cbl- $\beta$ -ligand transferase activity was shown to be reduced to 2–34% of the mean control value in four *cblC* cell lines but was also low (30%) in a *cblD* cell line (11). Thus the role of this enzyme in the *cblC* disorder remains to be confirmed. The *cblD* complementation group has so far only been assigned to two siblings (13) described in 1970 (14), whereas more than a hundred patients belonging to the *cblC* group have been reported (6). Studies of enzyme activities and *cbl* metabolism in fibroblasts of the *cblD* siblings revealed similar but less severe abnormalities compared with those found in *cblC* cells (15, 16).

Clinical presentation varies greatly both between and within the complementation groups. Patients with homocystinuria, isolated or in combination with MMA-emia, present with megaloblastic anemia and various neurological abnormalities (1, 17) and have, at least to some degree, responded biochemically and clinically to vitamin B<sub>12</sub> therapy. Patients with isolated MMA-emia usually present with metabolic decompensation and show lethargy, failure to thrive, feeding problems, and muscular hypotonia but no signs of megaloblastic anemia (6).

We present evidence for heterogeneity within the *cblD* complementation group based on biochemical findings and detailed enzyme studies in cultured skin fibroblasts in three new unrelated patients. In contrast to the previously reported *cblD* siblings, one patient revealed isolated MMA-uria, the other two isolated homocystinuria, although each belongs to the *cblD* complementation group when investigated by somatic complementation in cultured fibroblasts.

#### CASE REPORTS

Patient 1, born after uneventful pregnancy, was of Irish origin. Family relationships were complicated and suggested high grade consanguinity. At 6 years of age he presented with global developmental delay, severe learning difficulties and spastic ataxia, absence of ankle jerks, and rapid deterioration of gait. His weight and head circumference were at the 50th percentile. He had no vocal skills, made no eye contact, and on attempting to walk he held his arms abducted and flexed at the elbow. Reflexes were present in the upper limbs but absent in the lower limbs. A cranial MRI scan revealed cerebral and cerebellar atrophy. Visually evoked responses were poorly formed and delayed, and an electroretinogram analysis was normal. The mean corpuscular volume was elevated (94 fl; normal range, 70–87), but hemoglobin (11.9 g/dl) and plasma B<sub>12</sub> (787 ng/liter; normal range, 170–900) were normal. The folate content was elevated in the serum (20  $\mu$ g/liter; normal range, 1.6–13.2) and in red cells (1114  $\mu$ g/liter; normal range, 125–600). Plasma non-protein-bound homocystine was elevated (9  $\mu$ M; normal, not detectable), and methionine was low (14  $\mu$ M; normal range, 15–40). MMA was not elevated in urine. Treatment consisted of betaine (9–15 g/day), folic acid, which was later changed to folic acid (15 mg/day), and OH-Cbl (1 mg intramuscularly daily, later 1 mg weekly). After 1 week on therapy, plasma non-protein-bound homocystine was not detectable and the methionine level was normal (37  $\mu$ M). The patient appeared more alert and had increased muscle tone in all limbs with brisk tendon reflexes. Six months later he was able to walk and became very vocal.

At 16 years of age, compliance with medication was good, weight and height were between the 10th and 25th, and head circumference was at the 10th percentile. He had nearly normal gait, could speak a number of single words, but required help with dressing and washing; he could finger-feed and drink from a cup but is incontinent. He showed erratic behavior,

hyperactivity, and aggression, and his sleep pattern was poor. Although the intelligence quotient could not be formally assessed, the patient was clearly severely mentally retarded.

Patient 2 was the first child of healthy unrelated Italian parents. Pregnancy and delivery were unremarkable. At the age of 3 months he presented with severe hypotonia, nystagmus, dystonic movements, and seizures that were difficult to control with anticonvulsants. Brain MRI analysis showed reduced myelination and a small cerebellar vermis. Megaloblastic anemia (mean corpuscular volume, 105 fl; hemoglobin, 8.5 g/dl; and hematocrit, 25.4%) with normal plasma folate and slightly reduced B<sub>12</sub> (156 ng/liter; normal range, 200–1000) results were found. Plasma total homocystine was elevated (128  $\mu$ M; normal range, 5–16), and methionine decreased (4  $\mu$ M; normal range, 15–54). MMA was not elevated in urine.

Betaine (Cystadane®, Orphan Europe, 200 mg/kg/day), folic acid (15 mg/day), and OH-Cbl (1 mg/day) treatment resulted in clinical and biochemical improvement. Seizures disappeared within 10 days, and hematological status normalized. Plasma total homocystine decreased to 23  $\mu$ M, and methionine became normal (38  $\mu$ M). At the age of 3 years, betaine was stopped and OH-Cbl injections were reduced to 1 mg twice a week resulting in slightly higher values of plasma total homocystine (42  $\mu$ M). Reintroduction of betaine (100 mg/kg/day) lowered plasma total homocystine to 25  $\mu$ M.

At the age of 4 years his gross and fine motor skills were normal. An MRI showed normal development of the brain, however, speech delay was present and the intelligence quotient assessed by the Stanford-Binet scale was 84.

Patient 3 was the second child of unrelated parents of Indian origin, born at 32 weeks of gestation with a grade II respiratory distress syndrome requiring artificial ventilation. Further complications were grade I cranial hemorrhage, necrotizing enterocolitis, and neonatal convulsions. MMA (978 mmol/mol creatinine; normal, <4) and methylcitrate (197 mmol/mol creatinine; normal, not detectable) were markedly elevated in urine. Hemoglobin and differential blood count were normal. Therapy with intramuscular OH-Cbl (1 mg/week, later 2  $\times$  0.5 mg/week), carnitine, protein restriction, and phenobarbital resulted in normalization of metabolite excretion and the electroencephalogram. Non-protein-bound homocystine was not detected in the plasma, and later normal plasma total homocystine (8.9  $\mu$ M) was found even when treatment compliance was poor. Withdrawal of B<sub>12</sub> therapy resulted in an increase of metabolite excretion (MMA: 18,425 mmol/mol creatinine; methylcitrate: 67 mmol/mol creatinine), which was readily reversed upon reintroduction of protein restriction and B<sub>12</sub> therapy.

At the age of 12 year the patient attended a special school because of mild learning problems. Treatment continued as done previously but compliance was poor and clinic attendance was sporadic. Accordingly, MMA excretion was usually above 1000 mmol/mol creatinine, and his electroencephalogram was abnormal. The intelligence quotient was 69 (Hawik-III test).

#### EXPERIMENTAL PROCEDURES

**Cell Lines**—Fibroblasts were grown from skin biopsies obtained with informed consent of the patients or their parents. Cells were cultured in Earle's minimal essential medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and antibiotics as described earlier (18).

Reference cell lines belonging to different *cbl* mutant classes were characterized with methods described below and included five *cblA*, six *cblB*, five *cblC*, nine *cblE*, and six *cblG* cell lines. The complementation group of each of these cell lines was established by complementation analysis using defined cell lines obtained from the Montreal Cell Repository (*cblE* and *cblG* mutants) or cell lines in which complementation analysis was earlier performed in the laboratory of Prof. D. Rosenblatt (*cblA*, *cblB*, and *cblC* mutants). Cell lines WG1220 (one of the original siblings with *cblD* defect), designated here as the original *cblD* cell line, WG1575 (*cblE*), and WG1308 (*cblG*) were obtained from the Repository

TABLE I  
Activities of enzymes related to methylmalonic acid metabolism in cultured skin fibroblasts

Fibroblasts were grown in basal medium without (–) and with (+) 10 µg of hydroxocobalamin (OH-Cbl)/ml medium for propionate incorporation assay, and in basal medium without and with 1 µg of OH-Cbl/ml medium for methylmalonyl-CoA mutase assay. Methylmalonyl-CoA mutase was assayed without (holo-enzyme activity) and with (total enzyme activity) 50 µM adenosylcobalamin (AdoCbl). *n* = number of different control cell lines. The values for patients 1, 2, and 3 are the means of duplicate determinations from three individual experiments, the values for the original *cblD* cell line are the means of duplicate determinations from a representative experiment, and the values for the different *cbl* mutant cell lines and control cell lines are the mean and range of results obtained in each cell line in a representative experiment with duplicate determinations. Results of three OH-Cbl non-responsive and three OH-Cbl-responsive *cblB* cell lines are shown separately.

	Propionate incorporation		Methylmalonyl-CoA mutase				Adenosyltransferase
	nmol/16 h/mg protein		Holo-enzyme (–AdoCbl)		Total enzyme (+AdoCbl)		
	–	+	–	+	–	+	
OH-Cbl in medium →							
Patient 1	8.4, 9.8, 11	8.4, 9.5, 11	73, 93, 139	102, 135, 281	493, 753, 775	465, 875, 1185	3.1, 2.5, 1.9
Patient 2	6.2, 8.9, 9.4	6.4, 8.1, 11	90, 110, 117	104, 179, 192	938, 998, 765	919, 935, 778	0.71, 0.76, 1.2
Patient 3	1.3, 1.4, 1.9	16, 16, 14	12, 13, 78	46, 26, 23	1338, 2108, 554	916, 1446, 393	2.0, 2.5, 3.4
5 <i>cblA</i> cell lines	1.3 (0.73–2.0)	4.9 (2.8–7.4)	31 (18–45)	33 (13–69)	989 (544–1762)	247 (129–413)	1.9 (1.5–2.4)
3 <i>cblB</i> cell lines	1.4 (0.62–1.9)	3.6 (1.4–4.8)	26 (11–45)	34 (27–42)	1334 (884–1615)	845 (710–915)	0 (0–0.03)
3 <i>cblB</i> cell lines	1.0 (0.71–1.5)	1.2 (0.75–1.8)	38 (4.4–94)	36 (12–83)	1188 (554–1826)	786 (557–1086)	0 (0–0.001)
5 <i>cblC</i> cell lines	1.1 (0.24–1.8)	9.4 (2.5–19.6)	43 (15–95)	52 (30–127)	1178 (676–1627)	536 (150–813)	2.3 (1.8–3.2)
Original <i>cblD</i>	1.4	4.7	26	40	1376	828	2.8
9 <i>cblE</i> cell lines	10 (3.7–22)	10 (3.9–21)					
6 <i>cblG</i> cell lines	11 (5.3–17)	11 (5.8–18)					
Control cell lines	12 (6.4–18)	12 (5.2–21)	53 (24–106)	539 (265–1251)	1029 (575–2057)	1237 (585–2570)	1.2 (0.2–2.3)
<i>n</i>	13	13	24	24	24	24	12

for Mutant Human Cell Strains (The McGill University-Montreal Children's Hospital Research Institute, Montreal, Canada).

**Assays in Intact Fibroblast Monolayers**—Incorporation of [<sup>14</sup>C]propionate was assayed by a slight modification of the method described by Willard *et al.* (19), and formation of [<sup>14</sup>C]methionine from [<sup>14</sup>C]formate was determined as described earlier (20). Both assays were performed in intact fibroblasts grown in normal and OH-Cbl-supplemented media.

Uptake of CN-[<sup>57</sup>Co]Cbl and *cbl* coenzyme synthesis from CN-[<sup>57</sup>Co]Cbl was determined in intact fibroblasts as described earlier (21). CN-[<sup>57</sup>Co]Cbl was bound to transcobalamin in normal human serum by incubation for 30 min at 37 °C. Fibroblasts were incubated with medium containing this serum at a final concentration of 10% (v/v) for 4 days in the dark. Cells were harvested, disrupted by freezing and thawing, and *cbl* derivatives were extracted in hot ethanol and separated by high performance liquid chromatography.

**Specific Enzyme Assays**—Fibroblasts were harvested by trypsinization and cell free lysates prepared for enzyme assay. MMA-CoA mutase activity was assayed by measuring the conversion of [<sup>14</sup>C]MMA-CoA to [<sup>14</sup>C]succinyl-CoA in the presence (total mutase) and absence (holo-mutase) of 50 µM AdoCbl as described earlier (22). Methionine synthase activity was measured under high reducing conditions (dithiothreitol, 28 mM) by measuring the formation of [<sup>14</sup>C]methionine from [<sup>14</sup>C]methyltetrahydrofolate and L-homocysteine in the presence (total methionine synthase) and absence (holo-methionine synthase) of 50 µM MeCbl (modified from Ref. 23). These two assays were performed in fibroblasts cultured in normal and OH-Cbl supplemented (1 mg/liter) medium.

*Cbl* adenosyl transferase activity was determined by measuring the conversion of OH-[<sup>57</sup>Co]Cbl to Ado[<sup>57</sup>Co]Cbl in an H<sub>2</sub> atmosphere as described (24), except that the total OH-Cbl concentration in the assay mixture was 3.42 nM and OH-Cbl and AdoCbl were separated by high performance liquid chromatography as described for *cbl* coenzyme synthesis (see above). 5,10-Methylenetetrahydrofolate reductase (MTHFR) was assayed in its physiological forward direction in the presence of 75 µM flavin adenine dinucleotide as described earlier (18).

**Somatic Cell Complementation Analysis**—Complementation analysis was performed by a modification of an earlier described method (25). Briefly, heterokaryons were produced in mixed fibroblast cultures by treatment with 40% (v/v) polyethylene glycol 1500 (Sigma P-7181) for 120 s. Three days later incorporation of [<sup>14</sup>C]propionate or formation of [<sup>14</sup>C]methionine from [<sup>14</sup>C]formate was measured as described above. Within each experiment mixed unfused cells, self fusions, and fusion of cells from different known complementation groups were included as background, negative, and positive controls, respectively. In addition, patients' cells were fused with cell lines belonging to at least two different complementation groups to control the quality of the cells in each individual experiment. Further, the presence of fused cells, indicated by a characteristic enlarged cell structure, was confirmed by microscopic examination.

**Molecular Genetic Studies**—Genomic DNA and total RNA were extracted from cultured skin fibroblasts and cDNA was synthesized from RNA with standard protocols and kits (Invitrogen or Qiagen). The *MTR*

(*cblG*) and *MTRR* (*cblE*) genes, and genes shown to be implicated in the reductive activation of methionine synthase (genes coding for NR1 and cytochrome *b<sub>5</sub>*) were analyzed in patients 1 and 2 with isolated homocystinuria. The *MMAA* (*cblA*) gene was analyzed in patient 3 with isolated MMA-uria. Entire coding regions of the genes were PCR-amplified from cDNA and sequenced. Each identified genetic variant was verified at the genomic DNA level using PCR-restriction fragment length polymorphism or a PCR amplification refractory mutation system.

For analysis of the *MTR* gene cDNA containing the entire coding region (3798 bp) was amplified in four overlapping fragments (cDNA positions 65–1088/1027–2033/1921–2953/2887–3852). M13-specific sequence tags were attached to each primer in the 5'-position facilitating DNA sequencing using a subsequent PCR reaction. Automated sequence analysis was performed using an ABI Prism 3700 sequencer (Applied Biosystems) essentially as recommended by the manufacturer. The entire *MTRR* coding sequence with its flanking 5'- and 3'-untranslated regions was amplified in five overlapping segments employing cDNA and sequenced as described previously (25) except for a 5'-coding region (exons 1–5). This region contains alternative isoforms, and therefore exons 1–5 were analyzed by sequencing of PCR products derived from genomic DNA. The coding sequence of the *MMAA* gene was investigated by direct sequencing of PCR products derived from cDNA except for exons 6 and 7 (3'-coding region), which were analyzed by sequencing of PCR products derived from genomic DNA using published primers (8). The coding region of human *NR1* was investigated by direct sequencing of PCR products derived from two overlapping cDNA fragments using published primers (5). The coding region of human soluble cytochrome *b<sub>5</sub>* gene was investigated by direct sequencing in both directions of PCR products derived from cDNA using primers containing the 15 nucleotides specific to the soluble isoform (26).

## RESULTS

**Enzyme Activities, *cbl* Uptake, and *cbl* Coenzyme Synthesis**—Activities of enzymes related to MMA and homocysteine metabolism are shown in Tables I and II, respectively, and total *cbl* uptake and coenzyme synthesis results are given in Table III, in fibroblasts of the three patients, in reference cell lines belonging to different *cbl* complementation groups, and in control fibroblasts.

Incorporation of propionate in intact fibroblasts measures integrity of the propionate to the succinate pathway, which is an indirect measure of MMA-CoA mutase activity. Similarly, formation of methionine from formate in intact fibroblasts indirectly measures the activity of methionine synthase. These assays were performed in all cell lines to identify normal or defective function of the corresponding pathways.

**Enzyme Activities in Reference Cell Lines**—We define isolated

TABLE II  
Activities of enzymes related to homocysteine metabolism in cultured skin fibroblasts

For methionine formation and methionine synthase assays fibroblasts were grown in basal medium without (–) supplementation and with (+) 1  $\mu\text{g}$  of hydroxocobalamin (OH-Cbl)/ml. Methionine synthase was assayed without (holo-enzyme activity) and with (total enzyme activity) 50  $\mu\text{M}$  methylcobalamin (MeCbl). 5,10-Methylenetetrahydrofolate reductase (MTHFR) activity was assayed in the presence of 75  $\mu\text{M}$  flavin adenine dinucleotide. *n* = number of different control cell lines. The values for patients 1, 2, and 3 are the means of duplicate determinations from three individual experiments, the values for the original *cblD* cell line are the means of duplicate determinations from a representative experiment, and the values for the different *cbl* mutant cell lines and control cell lines are the mean and range of results obtained in each cell line in a representative experiment with duplicate determinations. Results of five OH-Cbl non-responsive and one OH-Cbl-responsive *cblG* cell line are shown separately.

	Methionine formation		Methionine synthase				MTHFR
			Holo-enzyme (–MeCbl)		Total enzyme (+MeCbl)		
	<i>nmol/16 h/mg protein</i>		<i>pmol/min/mg protein</i>				<i>nmol/h/mg</i>
OH-Cbl in medium →	–	+	–	+	–	+	–
Patient 1	0.50, 0.58, 0.66	1.2, 1.9, 2.7	0.7, 3.4, 6.6	9.9, 28, 28	22, 56, 78	64, 66, 101	19.9, 33.2, 38.6
Patient 2	0.21, 0.43, 0.65	1.2, 1.2, 1.5	3.7, 4.7, 8.3	16, 19, 24	26, 38, 39	43, 44, 58	13.5, 14.5, 23.3
Patient 3	2.2, 2.2, 3.1	2.0, 2.6, 3.2	26, 29, 49	189, 194, 220	118, 130, 144	338, 356, 381	17.9, 21.6, 24.6
5 <i>cblA</i> cell lines	3.2 (2.3–4.2)	3.7 (3.0–4.3)					
6 <i>cblB</i> cell lines	2.9 (2.4–3.4)	3.4 (2.9–4.2)					
5 <i>cblC</i> cell lines	0.07 (0.03–0.19)	2.2 (1.3–3.6)	1.1 (0–3.4)	46 (10–141)	48 (7.8–93)	77 (40–195)	23.6 (10.5–39.1)
Original <i>cblD</i>	0.38	0.84	8.1	20	47	53	23.6
9 <i>cblE</i> cell lines	0.10 (0.04–0.23)	0.14 (0.03–0.37)	45 (21–77)	163 (92–217)	99 (51–136)	230 (163–317)	23.9 (17.8–42.9)
5 <i>cblG</i> cell lines	0.22 (0.03–0.29)	0.23 (0.03–0.34)	4.5 (2.1–7.1)	13 (2.0–29)	21 (1.4–53)	41 (1.4–128)	26.6 (19.8–49.6)
1 <i>cblG</i> cell line	0.63, 0.97, 1.0	2.2, 2.3, 2.4	3.2	17	14	54	23.9
Control cell lines	2.5 (1.4–4.3)	2.9 (1.7–5.0)	32 (13–56)	223 (88–496)	81 (33–159)	348 (139–625)	25.9 (14.5–54.6)
<i>n</i>	10	10	16	16	16	16	75

TABLE III  
Total uptake of  $\text{CN-}^{57}\text{Co}$  cobalamin and cobalamin coenzyme synthesis in fibroblasts

The values for patients 1, 2, and 3 are the means of duplicate determinations from three individual experiments, the values for the original *cblD* cell line are the means of duplicate determinations from a representative experiment, and the values for the different *cbl* mutant cell lines and control cell lines are the mean and range of results obtained in each cell line in a representative experiment with duplicate determinations.

	Total uptake	Distribution of cobalamin <sup>a</sup>				
		CN-Cbl	OH-Cbl	AdoCbl	MeCbl	Others
	<i>pg/mg protein</i>			<i>% of total</i>		
Patient 1	34, 43, 56	12, 20, 24	7.5, 11, 8.1	77, 58, 65	2.9, 5.1, 3.3	0, 6.4, 0
Patient 2	62, 56, 57	23, 35, 24	16, 10, 5.8	55, 49, 65	5.6, 6.6, 5.0	0.6, 0, 0
Patient 3	76, 98, 76	17, 19, 20	7.8, 14, 9.2	8.2, 8.6, 5.3	67, 58, 66	0, 0, 0
5 <i>cblA</i> cell lines	58 (44–87)	20 (13–26)	17 (13–28)	7.7 (3.2–11)	55 (49–64)	0.2 (0–0.9)
6 <i>cblB</i> cell lines	64 (37–97)	22 (11–35)	18 (9.7–35)	7.0 (4.1–13)	53 (40–67)	0.1 (0–0.6)
5 <i>cblC</i> cell lines	9.1 (3.5–17)	73 (68–79)	16 (13–24)	6.4 (3.9–7.8)	3.5 (0–7.0)	1.0 (0–5.0)
Original <i>cblD</i>	17	46	30	10	14	0
9 <i>cblE</i> cell lines	54 (35–80)	26 (16–39)	34 (18–51)	25 (14–34)	12 (4.8–20)	3.7 (0–13)
6 <i>cblG</i> cell lines	64 (53–85)	13 (6.0–18)	22 (12–35)	58 (41–74)	5.6 (3.2–8.8)	1.8 (0–5.4)
Controls ( <i>n</i> = 23)	62 (39–119)	12 (6.0–17)	14 (4.7–28)	19 (14–26)	54 (39–69)	1.0 (0–5.6)

<sup>a</sup> CN-Cbl, cyanocobalamin; OH-Cbl, hydroxocobalamin; AdoCbl, adenosylcobalamin; MeCbl, methylcobalamin; *n*, number of different control cell lines.

functional deficiency of MMA-CoA mutase, as seen in *cblA* and *cblB* reference cell lines, as reduced incorporation of propionate, reduced synthesis of AdoCbl combined with normal MeCbl synthesis, and normal formation of methionine. Isolated functional deficiency of methionine synthase, as seen in *cblG* and *cblE* reference cell lines, was defined as reduced formation of methionine, reduced synthesis of MeCbl with normal or elevated synthesis of AdoCbl, and normal incorporation of propionate. All cell lines belonging to the *cblC* complementation group as well as the original *cblD* cell line showed combined functional deficiency of MMA-CoA mutase and methionine synthase.

*In vitro* OH-Cbl responsiveness of the different cell lines, evaluated by measuring incorporation of propionate or formation of methionine in cells grown in medium with added OH-Cbl (10 mg/liter and 1.0 mg/liter, respectively), is shown in Tables I and II. Responsiveness was defined as a reproducible, approximate doubling of activity or more, reaching at least 25% of the lowest control value in OH-Cbl supplemented medium compared with activity in basal medium. Thus, all *cblA* and *cblC* reference cell lines, three of the six *cblB* cell lines, the original *cblD* cell line, and one of the six *cblG* cell lines were OH-Cbl-responsive showing 2.3–10 times higher incorporation of propionate or 2.7–106 times higher formation of methionine. Levels of activities ranged from 27% to 381% of the lowest

control value. The other three *cblB* cell lines, five of the *cblG* cell lines, and all nine *cblE* cell lines showed no such response to OH-Cbl. In control cells, addition of OH-Cbl to the culture medium had no or only a minor effect (maximum 40% increase) on incorporation of propionate or formation of methionine. It should be noted that our enzyme data in the different reference cell lines, including the original *cblD* cell line, are similar to those reported by others (15, 27).

*Enzyme Activities in Fibroblasts of Patients 1 and 2*—Results of the enzyme assays clearly indicate isolated functional deficiency of methionine synthase in patients 1 and 2. Thus, in both patients' cells, formation of methionine and MeCbl synthesis were reduced whereas synthesis of AdoCbl was elevated and incorporation of propionate was normal. Accordingly, the specific activity of methionine synthase was reduced under all assay conditions, in cells grown in both basal and OH-Cbl supplemented medium (Table II). The specific activity of total MMA-CoA mutase was normal under all conditions. The relatively high holo-mutase activity in unsupplemented medium compared with controls may reflect the presence of elevated levels of AdoCbl (Table III). It should be noted that there is a tendency toward low holo-mutase activity in OH-Cbl-supplemented medium, the reason for which remains unclear. Fibroblasts of patients 1 and 2 showed a clear response to OH-Cbl

with on average 3.1 and 3.4 times higher formation of methionine, respectively, after growth with added OH-Cbl.

These findings were similar to those in the *cblG* cell lines, especially a single one responsive to OH-Cbl, but different from those in the *cblE* cell lines, which showed normal methionine synthase activity in the presence of added reducing agent, and were also not responsive to OH-Cbl. Levels of methionine synthesis in basal medium in both patients were higher than in *cblC* cell lines as also observed in the original *cblD* cell line. Total cbl uptake was clearly normal in contrast to the reduced values seen in *cblC* and the original *cblD* cells (Table III). The activity of MTHFR was normal in both cell lines (Table II). These findings provide clear evidence for an isolated defect of MeCbl synthesis without impaired synthesis of AdoCbl in both patients 1 and 2.

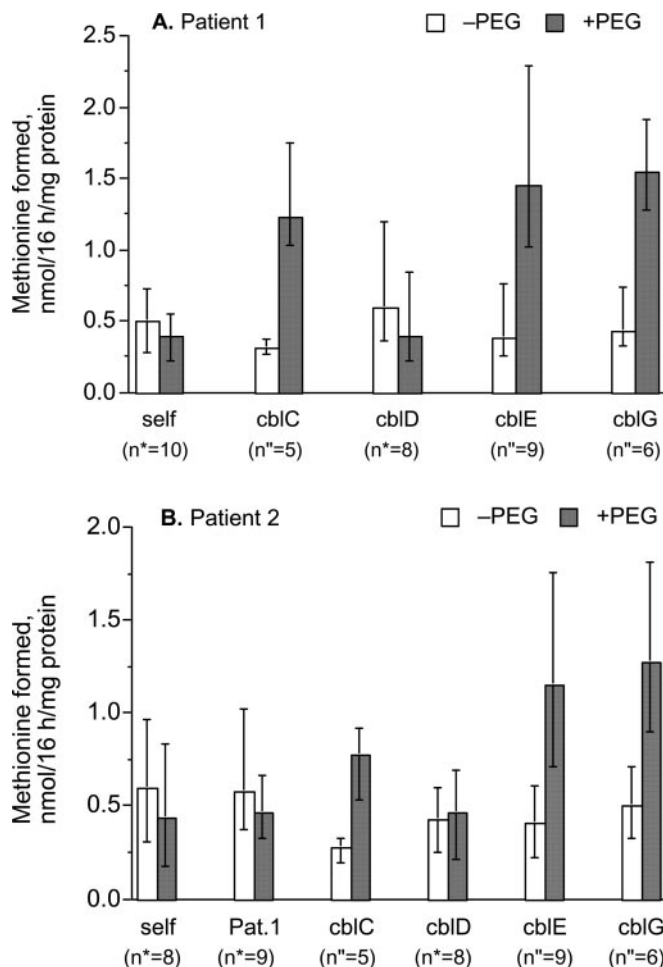
**Enzyme Activities in Fibroblasts of Patient 3**—Isolated functional deficiency of MMA-CoA mutase was indicated by reduced incorporation of propionate and AdoCbl synthesis but normal MeCbl synthesis and formation of methionine. Direct assay of MMA-CoA mutase revealed markedly reduced holomutase activity together with normal total mutase activities (Table I) in cells grown both in basal and in OH-Cbl-supplemented medium. Methionine synthase activity was normal under all assay and growth conditions (Table II).

Fibroblasts of patient 3 showed a dramatic response to OH-Cbl with on average 10 times higher incorporation of propionate after growth with OH-Cbl, values exceeding the mean control value (Table I). This, together with clearly normal total mutase activity, makes a mild form of MMA-CoA mutase apoenzyme highly unlikely, because the highest total mutase activity observed in 80 mut cell lines, including 11 with the  $\text{mut}^-$  defect, was less than 50% of the lowest control value (results not shown).

Comparison of enzyme activities allowed no distinction between cells of patient 3 and *cblA* except for low total mutase activity in OH-Cbl-supplemented medium in the *cblA* cells but not in patient 3. Cells of patient 3 could be clearly distinguished from the OH-Cbl-responsive *cblB* cell lines by the presence of normal cbl adenosyltransferase activity (Table I). These findings provide clear evidence for an isolated defect of AdoCbl synthesis without impaired MeCbl synthesis in patient 3.

**Molecular Genetic Studies**—Sequencing of the entire coding regions of the *MTR*, *MTRR*, *NR1*, and cytochrome  $b_5$  genes in patients 1 and 2, and of the *MMAA* gene in patient 3 revealed no obvious pathogenic mutant alleles. A number of polymorphic changes were observed as follows.

The missense mutation c.940G>A (D314N) of the *MTR* gene was observed in patient 1 in the homozygous and in patient 2 in the heterozygous state and is known to be a polymorphism.<sup>2</sup> Also in patient 1, three homozygous synonymous changes (c.3144A>G, c.3492A>C, and c.3576C>T) of the *MTR* gene, and nine heterozygous SNPs of the *MTRR* gene were identified, all of which are known polymorphisms (28).<sup>2</sup> Of the nine SNPs of the *MTRR* gene two are missense mutations (c.66A>G and c.1049A>G), five are synonymous changes (c.537T>C, c.1464A>G, c.1536C>T, c.1761T>C, and c.1875G>A), and two were detected in the 3'-untranslated region of the gene (c.2146A>G and c.2248A>G). This combination of SNPs in one haplotype is unlikely to be pathogenic, because the same combination was also identified in control chromosomes (data not shown). In patient 2 heterozygosity for the known polymorphism (c.2756A>G) of the *MTR* gene (28) and homozygosity for one genetic variant (c.66A>G) of the *MTRR* gene were found. No polymorphisms were detected in the coding regions of the



**FIG. 1. Somatic complementation of fibroblasts of patients 1 (A) and 2 (B) with five *cblC*, the original *cblD*, nine *cblE*, and six *cblG* cell lines.** Fibroblasts of the patients were mixed with fibroblasts belonging to these known complementation groups, fused with polyethylene glycol 1500 (PEG) treatment (shaded columns), and 3 days later formation of methionine was measured as described under "Experimental Procedures." Parallel cultures of mixed unfused cells were used as background controls (unshaded columns). Self-fusions were used as a negative control. Activities are the mean and the range (vertical lines) of results from either different experiments with single determinations ( $n^*$  = number of individual experiments) or of results obtained with different cell lines ( $n'$  = number of different cell lines). Each result with a different cell line is the mean of results obtained in two individual experiments with a single determination.

*MMAA*, the *NR1*, or the cytochrome  $b_5$  genes.

**Somatic Complementation Studies**—Fig. 1 shows the results of somatic complementation analysis, based on formation of methionine, with cells of patient 1 and 2 tested with five *cblC*, nine *cblE*, and six *cblG* cell lines, and the original *cblD* cell line. The mean level of formation of methionine was 3.5–3.9 times higher in cells of patient 1 and 2.6–2.8 times higher in cells of patient 2 when fused with *cblC*, *cblG*, or *cblE* reference cell lines as compared with mixed, unfused cells. In contrast, there was no clear increase after self-fusion, fusion of fibroblasts of the two patients with each other, or, importantly, after fusion of fibroblasts of both patients with the original *cblD* cell line. Thus, cells of both patients complemented all other *cbl* mutant cell lines except the original *cblD* cell line allowing assignment of patients 1 and 2 to the *cblD* complementation group, and we define them as *cblD*-variant 1.

Fig. 2 shows the results of somatic complementation analysis, based on incorporation of propionate, with cells of patient 3 tested together with five *cblA*, six *cblB*, and five *cblC*

<sup>2</sup> Dr. B. Janosikova, personal communication

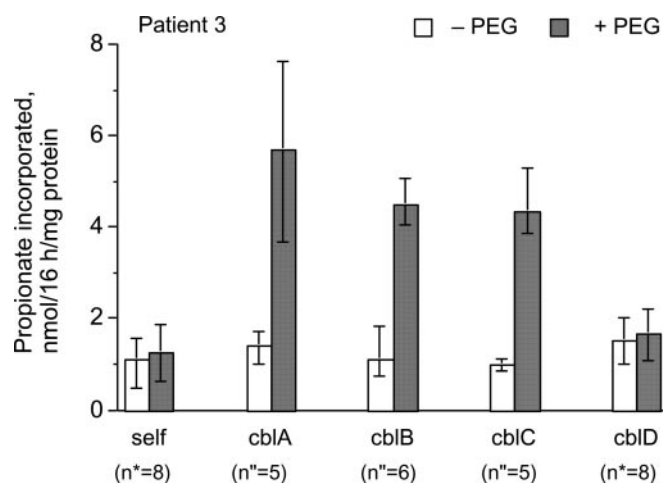


FIG. 2. Somatic complementation of fibroblasts of patient 3 with five *cblA*, six *cblB*, and five *cblC* cell lines as well as the original *cblD* cell line. Complementation was detected by the assay of the incorporation of propionate 3 days after fusion. For details see legend to Fig. 1.

cell lines and the original *cblD* cell line. The mean level of incorporation of propionate was 4.0–4.3 times higher after fusion with *cblA*, *cblB*, and *cblC* reference cell lines as compared with mixed, unfused cells. In contrast self-fusion and, importantly, fusion with the original *cblD* cell line led to no clear increase of propionate incorporation (maximum, 10% increase). Thus, cells of patient 3 complemented all other cell lines investigated except the original *cblD* cell line allowing assignment of patient 3 to the *cblD* complementation group, and we define it as *cblD*-variant 2.

#### DISCUSSION

Our study in three new unrelated *cblD* patients with isolated deficiency of either MeCbl or AdoCbl synthesis provides evidence for heterogeneity within the *cblD* mutant class, which was so far associated with combined deficiency of synthesis of both cofactors (1). Somatic complementation studies revealed that cells from patients 1 and 2 with isolated deficiency of MeCbl synthesis clearly complemented reference cell lines with this biochemical phenotype, namely *cblE* and *cblG*. Similarly, cells from patient 3 with isolated deficiency of AdoCbl synthesis complemented reference cell lines with the same biochemical phenotype, *i.e.* *cblA* and *cblB*. Expanding the complementation analysis with these three patients to include *cblC* reference cell lines and the original *cblD* cell line, both with combined deficiency of MeCbl and AdoCbl synthesis, revealed clear complementation with *cblC* cell lines, but no complementation with the original *cblD* cell line in repeated experiments. This conclusively indicates that each of these 3 patients belongs to the *cblD* complementation group, and they are designated as *cblD*-variant 1 (patients 1 and 2) and *cblD*-variant 2 (patient 3). A summary of our present understanding of the cellular and metabolic pathways involved in B12 coenzyme biosynthesis is shown in Fig. 3.

The *cblD* defect has been described in just two siblings in whom combined functional deficiency of MMA-CoA mutase and methionine synthase was demonstrated in cultured skin fibroblasts (15). The older sibling presented with developmental delay and neurological abnormalities at the age of 14 years at which time his 1.5-year-old affected sibling was apparently healthy (14). Both siblings showed discrete signs of megaloblastic anemia when studied later, at the ages of 26 and 13 years, respectively (29). In contrast to the combined defect in these original siblings, detailed studies in fibroblasts of our patients 1 and 2 provide clear evidence of an isolated defect of methio-

nine synthase. Accordingly, they presented with neurological abnormalities and megaloblastic anemia without metabolic decompensation. Further, isolated functional MMA-CoA mutase deficiency was confirmed in fibroblasts from our patient 3 who presented with massive MMA-uria with normal plasma total homocysteine and clinical signs compatible with MMA-emia.

Our study indicates that *cblD* mutants are not as rare as reported. Thus, we detected the two *cblD*-variant 1 cell lines among 17 with isolated functional methionine synthase deficiency, and one *cblD* variant 2 cell line by investigation of 22 patients suspected to have the *cblA* defect.

It has been pointed out that biochemical differences between the *cblC* and the original *cblD* cell lines are only quantitative and that the two mutant classes are only distinguished by complementation analysis (16). Since this original publication no further *cblD* patients have been reported, whereas more than one hundred patients belonging to the *cblC* complementation group have been described (1). Rosenblatt and Fenton (1) raised the possibility that the mutation in the *cblD* defect belongs to the same allele as in *cblC* and that the observed complementation is interallelic. Interallelic complementation has been shown to occur between different mutants of multimeric proteins and can be recognized by the occurrence of complementation between a variable number but not all cell lines of the same mutant class. For example, complementation analysis of 28 cell lines with mutations of the homotetrameric enzyme argininosuccinate lyase revealed 20 cell lines that complemented between 1 and 14 of the other cell lines (30). Similarly, within 10 cell lines with deficiency of the homodimeric MMA-CoA mutase (*mut0*), 8 complemented 1 to 6 of the others (31). In contrast to these and other examples, our 2 *cblD*-variant 1 cell lines clearly complemented each of a minimum of 5 cell lines of the *cblC*, *cblG*, and *cblE* classes. Also the *cblD*-variant 2 cell line complemented each of a minimum of 5 cell lines of the *cblA*, *cblB*, and *cblC* classes. Therefore our findings support the idea of *cblD* as a separate entity, although interallelic complementation cannot be completely excluded.

Further evidence that patients 1 and 2, and patient 3, do not belong to the known complementation groups *cblE* and *cblG*, or *cblA*, respectively, was provided by molecular genetic analysis of the coding region of the relevant genes. Thus sequencing of the *MTRR* (*cblE*) and *MTR* (*cblG*) genes in patients 1 and 2 revealed no potentially disease-causing mutations. Also, no mutations in the coding region of the *MMAA* gene (*cblA*) were detected in patient 3.

In addition, Olteanu and Banerjee (5) recently reported that human NR1 can activate methionine synthase in the presence of soluble cytochrome *b<sub>5</sub>* and suggested that this enzyme might fulfill a physiological function. Based on our previous observation that patient 1 cells complemented both *cblE* and *cblG* cell lines (32), they suggested that a mutation in the NR1/soluble cytochrome *b<sub>5</sub>* system could be the cause of the defect in patient 1. However, sequencing of the coding regions of the *NR1* and soluble cytochrome *b<sub>5</sub>* genes in patient 1 and 2 did not reveal any mutations. Therefore, it is highly unlikely that either NR1 or cytochrome *b<sub>5</sub>* is the protein responsible for the *cblD* complementation group.

In summary, we showed that none of the newly diagnosed *cblD* patients carry pathogenic mutations in the tested genes as listed above, thus strongly limiting the possibility that these genes play a role in the pathogenesis of the *cblD* complementation group. Of course the presence of pathogenic mutations in regulatory non-coding regions such as a promoter, which in rare cases can cause disease, cannot be excluded.

The only known complementation groups related to intracellular *cbl* metabolism that were not investigated in this study

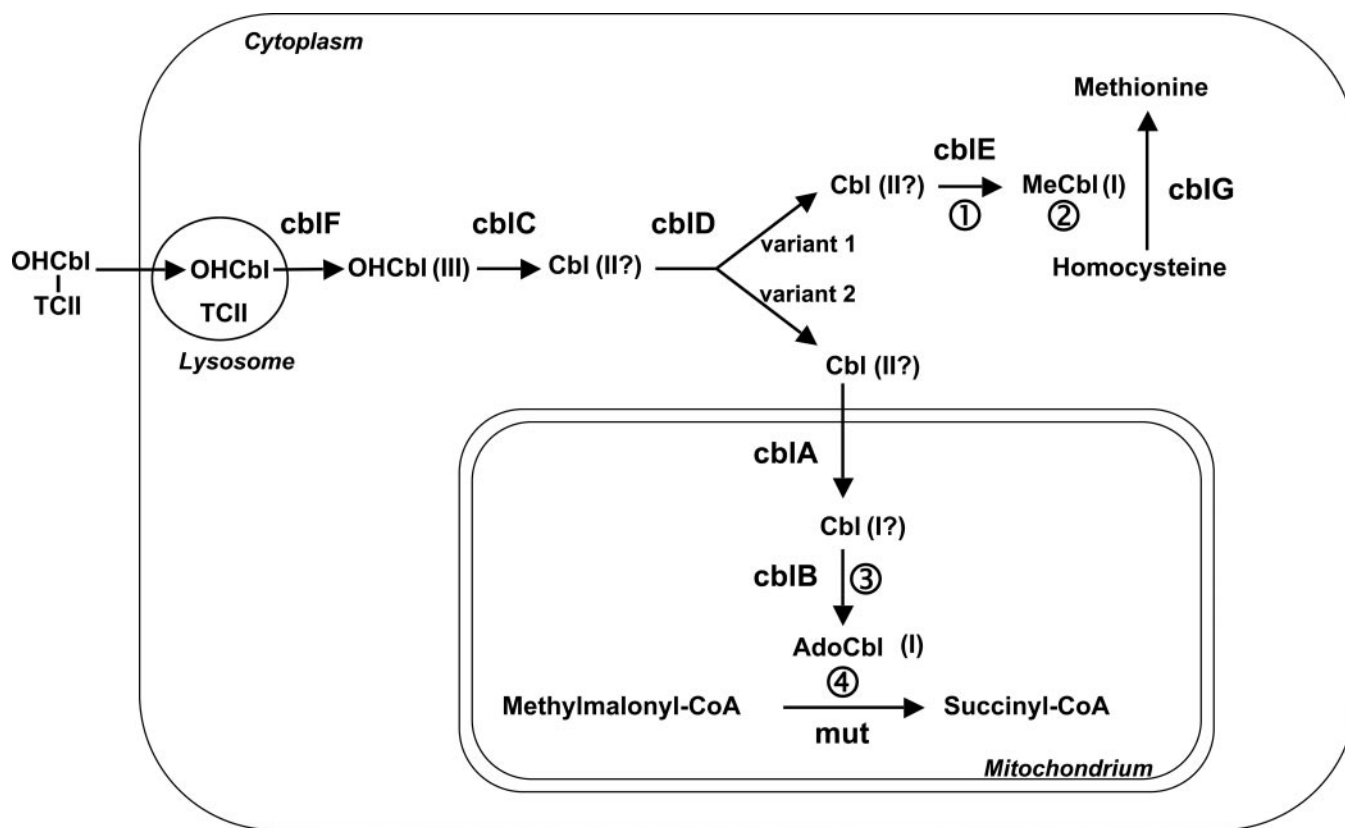


FIG. 3. Summary of intracellular processing of cobalamin. *Cbl*, cobalamin; *TCII*, transcobalamin; *OHCbl*, hydroxocobalamin; *MeCbl*, methylcobalamin; *AdoCbl*, adenosylcobalamin; *cblA*–*cblG* and *mut*, sites of defect in the various complementation groups as defined in the text; *variant 1* and *variant 2*, proposed sites of the *cblD* variants described in this study. The valence state of the cobalt atom of cobalamin is shown in parentheses, with “?” when not fully understood. Known enzymes are shown as follows: ①, methionine synthase reductase; ②, methionine synthase; ③, cobalamin adenosyltransferase; ④, methylmalonyl-CoA mutase.

are *cblF* and *cblH*. The *cblF* defect is a rare disorder of *cbl* release from the lysosomes leading to combined deficiency of methionine synthase and MMA-CoA mutase function as also seen in *cblC* reference cell lines (1, 10). A key feature of the *cblF* defect, also found in our hands (data not shown), is elevated total uptake of *cbl* reflecting its accumulation in lysosomes, in the presence of deficient synthesis of both MeCbl and AdoCbl. These findings clearly differ from those in our *cblD* variant cell lines, and therefore this complementation group was not included in this study.

The *cblH* complementation group was recently described by Watkins *et al.* (7) in a single cell line from a patient with isolated MMA-uria in which enzyme studies revealed isolated functional deficiency of MMA-CoA mutase, as also seen in patient 3. This cell line was not available to us. Assignment of their patient to a new mutant class was based on complementation with *mut*, *cblB*, *cblC*, *cblF*, and several *cblA* cell lines but not *cblD* (7, 27), raising the possibility that the newly described *cblH* cell line could in fact belong to the *cblD* class of mutants.

The gene responsible for the *cblD* complementation group as well as the function of the corresponding protein remain unknown. However, this study shows that mutations in such a protein most likely cause combined as well as isolated deficiencies of both AdoCbl and MeCbl synthesis. It could be speculated that the systems, leading to synthesis of MeCbl and AdoCbl, have different affinities for the *cbl* moiety produced by the *cblD* protein. Then residual activity of the *cblD* protein may produce sufficient product for the higher affinity, but not for the lower affinity system thereby leading to sufficient synthesis of one *cbl* coenzyme but not the other. However, this would explain the existence of the combined defect and only one of the isolated defects. A more likely explanation is that the *cblD* protein

contains at least three different domains, one *cbl* binding site, one required for the “presentation” of the reduced *cbl* to cytosolic methionine synthase and one needed for presentation to a mitochondrial membrane component. Thus, mutations within the *cbl* binding domain or those leading to complete disruption of the protein could result in reduced synthesis of both MeCbl and AdoCbl as seen in the original *cblD* cell line. Mutations within the methionine synthase recognition domain would affect only the synthesis of MeCbl, whereas the *cbl* would be available for AdoCbl synthesis as seen in the *cblD*-variant 1 cell lines, and, accordingly, mutations within the mitochondrial recognition domain would result in isolated deficiency of AdoCbl synthesis as seen in *cblD*-variant 2, as illustrated in Fig. 3. Another possible mechanism is that mitochondrial and cytosolic isoforms of the *cblD* protein exist that are formed by alternative splicing of a single gene. Thus mutations affecting a shared functionally important domain would cause the combined deficiency, whereas mutations causing defects in the cytosolic form or in the mitochondrial isoform or mitochondrial leader sequence would cause the two isolated deficiencies.

Finally, the unlikely possibility that the original *cblD* patients, who are consanguineous, have a defect in two independent genes cannot be ruled out. In this event one gene would be deficient in variant 1 and the other in variant 2. This possibility could have been ruled out by complementation analysis of variants 1 and 2 with each other, but none of the available methods revealed a common functional deficiency precluding such an experiment.

In conclusion, our study shows that the *cblD* complementation group contains not only patients with combined deficiency of the synthesis of both MeCbl and AdoCbl but also patients with isolated deficiency of either MeCbl or AdoCbl synthesis.

However, the protein responsible for the *cblD* group as well as its primary function remains to be detected. Our findings help to delineate the pathways of intracellular *cbl* metabolism and will help in identification of the so far unidentified corresponding genes and proteins.

*Acknowledgment*—We thank Mira Günther for her excellent assistance in the enzyme assays.

## REFERENCES

- Rosenblatt, D. S., and Fenton, W. A. (2001) in *The Metabolic and Molecular Bases of Inherited Disease* (Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., eds) 8th Ed., pp. 3897–3933, McGraw-Hill, Inc., New York
- Gulati, S., Baker, P., Li, Y. N., Fowler, B., Kruger, W., Brody, L. C., and Banerjee, R. (1996) *Hum. Mol. Genet.* **5**, 1859–1865
- Leclerc, D., Wilson, A., Dumas, R., Gafuik, C., Song, D., Watkins, D., Heng, H. H. Q., Rommens, J. M., Scherer, S. W., Rosenblatt, D. S., Rozen, R., and Gravel, R. A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 3059–3064
- Watkins, D., and Rosenblatt, D. S. (1988) *J. Clin. Invest.* **81**, 1690–1694
- Olteanu, H., and Banerjee, R. (2003) *J. Biol. Chem.* **278**, 38310–38314
- Fenton, W. A., Gravel, R. A., and Rosenblatt, D. S. (2001) in *The Metabolic and Molecular Bases of Inherited Disease* (Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., eds) 8th Ed., pp. 2165–2193, McGraw-Hill, Inc., New York
- Watkins, D., Matiaszuk, N., and Rosenblatt, D. S. (2000) *J. Med. Genet.* **37**, 510–513
- Dobson, C. M., Wai, T., Leclerc, D., Wilson, A., Wu, X., Dorè, C., Hudson, T., Rosenblatt, D. S., and Gravel, R. A. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 15554–15559
- Dobson, C. M., Wai, T., Leclerc, D., Kadir, H., Narang, M., Lerner-Ellis, J. P., Hudson, T. J., Rosenblatt, D. S., and Gravel, R. A. (2002) *Hum. Mol. Genet.* **26**, 3361–3369
- Watkins, D., and Rosenblatt, D. S. (1986) *Am. J. Hum. Genet.* **39**, 404–408
- Pezacka, E. H. (1993) *Biochim. Biophys. Acta* **1157**, 167–177
- Watanabe, F., Saido, H., Yamaji, R., Miyatake, K., Isegawa, Y., Ito, A., Yubisui, T., Rosenblatt, D. S., and Nakano, Y. (1996) *J. Nutr.* **126**, 2947–2951
- Willard, H. F., Mellman, I. S., and Rosenberg, L. E. (1978) *Am. J. Hum. Genet.* **30**, 1–13
- Goodman, S. I., Moe, P. G., Hammond, K. B., Mudd, S. H., and Uhlenhuth, B. W. (1970) *Biochem. Med.* **4**, 500–515
- Fenton, W. A., and Rosenberg, L. E. (1978) *Annu. Rev. Genet.* **12**, 223–248
- Mellman, I. S., Willard, H. F., Youngdahl-Turner, P., and Rosenberg, L. E. (1979) *J. Biol. Chem.* **254**, 11847–11853
- Watkins, D., and Rosenblatt, D. S. (1989) *Am. J. Med. Genet.* **34**, 427–434
- Suormala, T., Gamse, G., and Fowler, B. (2002) *Clin. Chem.* **48**, 835–843
- Willard, H. F., Ambani, L. M., Hart, A. C., Mahoney, M. J., and Rosenberg, L. E. (1976) *Hum. Genet.* **34**, 277–283
- Fowler, B., Whitehouse, C., Wenzel, F., and Wraith, J. E. (1997) *Pediatr. Res.* **41**, 145–151
- Fowler, B., and Jakobs, C. (1998) *Eur. J. Pediatr.* **157**, 88–93
- Baumgartner, R. (1983) in *The Cobalamins: Methods in Hematology* (Hall, C. A., ed) Vol. 10, pp. 181–195, Churchill Livingstone, Edinburgh, New York
- Mellman, I., Willard, H. F., and Rosenberg, L. E. (1978) *J. Clin. Invest.* **62**, 952–960
- Fenton, W. A., and Rosenberg, L. E. (1981) *Biochem. Biophys. Res. Commun.* **98**, 283–289
- Zavadakova, P., Fowler, B., Zeman, J., Suormala, T., Pristoupilova, K., and Kozich, V. (2002) *J. Inher. Metab. Dis.* **25**, 461–476
- Giordano, S. J., and Steggle, A. W. (1991) *Biochem. Biophys. Res. Commun.* **178**, 38–44
- Cooper, B. A., Rosenblatt, D. S., and Watkins, D. (1990) *Am. J. Hematol.* **34**, 115–120
- Watkins, D., Ru, M., Hwang, H. Y., Kim, C. D., Murray, A., Philip, N. S., Kim, W., Legakis, H., Wai, T., Hilton, J. H., Ge, B., Dore, C., Hosack, A., Wilson, A., Gravel, R. A., Shane, B., Hudson, T. J., and Rosenblatt, D. S. (2002) *Am. J. Hum. Genet.* **71**, 143–153
- Carmel, R., and Goodman, S. I. (1982) *Blood* **59**, 306–311
- McInnes, R. R., Shih, V., and Chilton, S. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81**, 4480–4484
- Qureshi, A. A., Crane, A. M., Matiaszuk, N. V., Rezvani, I., Ledley, F. D., and Rosenblatt, D. S. (1994) *J. Clin. Invest.* **93**, 1812–1819
- Fowler, B., Suormala, T., Günther, M., Till, J., and Wraith, J. E. (1997) *J. Inher. Metab. Dis.* **20**, Suppl. 1, 21 (abstract)