The molecular characterization of inborn errors of vitamin B_{12} metabolism: *cblA*, *cblB* and *cblC*

By

Jordan Lerner-Ellis
Department of Human Genetics
McGill University, Montreal, Canada

A thesis submitted to McGill University in partial fulfilment of the requirements of the degree of Doctor of Philosophy

Your file Votre référence
ISBN: 978-0-494-32368-7
Our file Notre référence
ISBN: 978-0-494-32368-7

ABSTRACT

This work investigates the molecular basis of three genetic diseases of vitamin B_{12} metabolism: *cblA*, *cblB* and *cblC*. Two genes responsible for isolated forms of methylmalonic aciduria types *cblA* and *cblB*, called *MMAA* and *MMAB* respectively, were recently identified. We sequenced the coding sequence and flanking regions of the *MMAA* and *MMAB* genes from the gDNA of 37 *cblA* and 35 *cblB* patient cell lines and identified 31 novel mutations in total. The biochemical properties of these cell lines were examined in cell culture. Haplotype analysis was used to investigate the history of mutations. The occurrence of both rare and common mutations were identified. Attempts were made to make geneotype-phenotype correlations and to understand the effects of mutations on protein function. In the *MMAA* gene 18 novel mutations were identified, eight of which were common to two or more individuals. One mutation, c.433C>T represented 43% of pathogenic alleles and a common haplotype was identified. Diagnostic tests were designed for every mutation identified. In the *MMAB* gene, 13 novel mutations were identified. Most mutations were clustered in exon 7. One mutation, c.556C>T accounted for 33% of pathogenic alleles, associated with disease presentation in the first year of life, was observed on a common haplotype and seen almost exclusively in European individuals. We used a combination of linkage, sibling pair, homozygosity mapping and haplotype analyses to refine the disease locus and identify the gene responsible for *cblC* disease on chromosome 1p called *MMACHC*. We examined the gDNA of 204 *cblC* patient cell lines and identified 42 different mutations. The large number of patient samples allowed for the identification of specific genotype phenotype correlations. Of the mutations with elevated frequency in the patients examined, the c.271dupA and c.331C>T mutations were associated with early onset disease wherease c.394C>T was associated with late onset disease. Other missense mutations were also associated with onset of disease later in life. Seven mutations showed clustering by ethnicity. Eight SNPs were identified spanning the gDNA of the *MMACHC* gene and allowing for the identification of specific haplotypes and the determination of recurrent vs common mutations. Infection of the wild-type *MMACHC* gene into *cblC* patient fibroblast cell lines showed correction of the cellular phenotype. Examination of EST databases and northern blot analysis demonstrated *MMACHC* is ubiquitously expressed in

humans with higher levels in fetal liver. Multiple sequence alignment of genomic DNA in eukaryotes and of the polypeptide sequence demonstrated that MMACHC is well conserved in eukaryotes. Two functional domains were identified in the *MMACHC* gene product by comparison with bacterial genes involved in vitamin B_{12} related functions: a putative vitamin B_{12} binding domain and a TonB-like domain. Molecular modeling demonstrated that the C-terminal region of the gene product folds similarly to TonB from *E. coli* and suggesting that the C-terminal region of MMACHC may function in a simlar manner.

RÉSUMÉ

Ce travail fait l'investigation des fondements moléculaires de trois maladies génétiques du métabolisme de la vitamine B_{12}: cblA, cblB et cblC. Deux gènes responsables de l'acidurie méthylmalonique isolée de type cblA et cblB, désignés MMAA et MMAB respectivement, ont été récemment identifiés. Nous avons déterminé les séquences codantes et adjacentes des gènes MMAA et MMAB à partir de l'ADN génomique de 37 lignées cellulaires de patients cblA et 35 de patients cblB , et avons identifié 31 nouvelles mutations. Les propriétés biochimiques de ces lignées cellulaires ont été examinées en culture cellulaire. L'analyse des haplotypes a été utilisée pour investiguer l'histoire des mutations et l'occurrence à la fois de mutations rares et communes a été confirmée. Des essais ont été faits pour trouver une corrélation génotype-phénotype et pour comprendre les effets des mutations sur la fonction des protéines. Pour le gène MMAA, 18 nouvelles mutations ont été identifiées, dont 8 sont communes à deux individus ou plus. L'une des mutations, c.433C>T, représente 43% des allèles pathogènes et un haplotype commun a été identifié. Pour le gène MMAB, 13 nouvelles mutations ont été identifiées. La plupart des mutations sont situées dans l'exon 7. L'une des mutations, c.556C>T, représente 33% des allèles pathogènes et est associée avec l'apparition de la maladie durant la première année de vie. Cette mutation a été observée sur un haplotype commun et presque exclusivement sur des individus d'origine européenne. Nous avons utilisé une combinaison d'analyse de liaison, de paires fraternelles, de cartographie dite homozygosity mapping et d'analyse des haplotypes pour raffiner le locus de la maladie et identifier le gène responsable de la maladie de type cblC, MMACHC, sur le chromosome 1p. Nous avons examiné l'ADN génomique de 204 lignées cellulaires de patients cblC et identifié 42 différentes mutations. Le grand nombre d'échantillons de patients a permis l'identification de corrélations génotype-phénotype spécifiques. Parmi les mutations ayant une fréquence accrue chez les patients examinés, les mutations c.271dupA et c.331C>T sont associées avec une apparition hâtive des symptômes tandis que c.394C>T est associée avec une apparition tardive des symptômes. D'autres mutations sont aussi associées avec une apparition tardive des symptômes. Sept mutations ont démontré une répartition par ethnicité. Huit SNP ont été identifiés couvrant l'ADN génomique du gène MMACHC et ont permis l'identification d'haplotypes spécifiques et la détermination de

mutations récurrentes et communes. La transfection du gène MMACHC sauvage dans des lignées cellulaires de patient cblC a démontré une correction du phénotype. L'examen des bases de données d'EST et l'analyse d'immunobuvardage de type Northern ont démontré que le gène MMACHC est exprimé de façon omniprésente chez l'humain, avec un niveau accru dans le foie foetal. L'alignement de multiples séquences d'ADN génomique eucaryotique et de séquences de polypeptides a démontré que MMACHC est bien conservé chez les eucaryotes. Deux domaines fonctionnels ont été identifiés dans le gène MMACHC par comparaison avec des gènes bactériens ayant des fonctions reliées à la vitamine B_{12} : un domaine de liaison à la vitamine B_{12} qui apparaît être bien conservé à travers l'évolution et un domaine arborant une ressemblance à TonB, moins fidèlement conservé. Un modelage moléculaire a démontré que la région C-terminale du produit génique se replie similairement à TonB de *E. coli*, suggérant que la région C-terminale de MMACHC puisse fonctionner d'une manière analogue à TonB de *E. coli*.

ACKNOWLEDGEMENTS

To David S. Rosenblatt who has been more than just an experienced and trusted adviser; thank you for the kind support over the course of my PhD work.

To my family and friends who have been so supportive of me, especially Tasha Cyr who has helped me every step of the way.

This work is dedicated to the memory of Jamie Tirone whose time came too soon and who will always be missed. This work goes out to the families of individuals afflicted with inborn errors of vitamin B_{12} metabolism and with many thanks to all the physicians and scientists who participated in my work.

I would like to thank in particular David Watkins, Angela Hosack, Mary Fujiwara and Kenneth Morgan for many hours of discussions, hard work and support.

Thanks to Eric Shoubridge, Rima Rozen, Ken Dewar, Thomas Hudson, Johanna Rommens for your guidance and supervision.

I am grateful to all the members of the McGill University and Genome Quebec Innovation Centre who have participated in this work including, among others, Pierre Lepage, Carole Doré, Vince Forgetta, Amélie Villeneuve, David Roquis, Donna Sinnett, Corine Zotti, Gary Leveque, Scott Gurd, Andre Verner, Geneviève Genaud, Robert Sladek, and Alexendre Montpetit. Thank you to Peter Pawelek, Chris Ng Thow Hing, and James Coulton for helpful collaboration and instructions.

To all the technical staff, students and trainees of the Rosenblatt laboratory, including Gail Dunbar, Chantal Morel, Jocelyne Lavallée, Nora Matiaszuk, Brian Gilfix, Maria Galvez, Abigail Gradinger, Emily Moras, Junhui Liu, Amanda Loewy, Sabrina Selway, and Lama Yamani, thank you very much for your contributions and for your friendship.

Special thanks to Isabelle Racine Miousse and Danielle Malo for translation of the French abstract.

Many thanks to Yasmin Karim, Vanessa Flannery, Fran Langton, Laura Benner, and Kandace Springer for all your help during my graduate studies.

I would like to acknowledge the financial support for my graduate studies from the Canadian Institutes for Health Research, the McGill University Health Centre Research Institute, and the McGill University Faculty of Medicine.

CONTRIBUTIONS OF AUTHORS

Chapter 2.1:

Lerner-Ellis J.P: Writing, DNA extraction, PCR, Sequencing, primer design, genotype-phenotype correlations, analysis of cell culture studies, mutation annotation, data analysis.

Dobson CM: Primer design, mutation nomenclature, description of first mutations in the *MMAA* gene.

Wai T: DNA extraction, PCR and sequencing, description of first mutations.

Watkins D: Cell culture studies, data analysis, copy editor.

Tirone JC: Haplotype analysis, mutation confirmation, copy editor.

Leclerc D: Mutation nomenclature, copy editor.

Doré C: Sequencing and sequence analysis.

Lepage P: Sequence analysis.

Gravel RA: Copy editor.

Rosenblatt DS: Principal investigator.

Chapter 3.1:

Lerner-Ellis JP: Writing, DNA extraction, PCR, sequencing, mutation analysis, haplotype and data analyses.

Gradinger AB: PCR, sequencing, mutation analysis.

Watkins D: Cell culture studies, biochemical analyses, mutation analysis, haplotype analysis, manuscript writing and editing.

Tirone JC: Mutation confirmation.

Villeneuve A: Sequencing and sequence analysis.

Dobson CM: Identification of first mutations.

Montpetit A: Haplotype analysis, analysis of HapMap data.

Lepage P: Sequencing and sequence analysis.

Gravel RA: Copy editor.

Rosenblatt: Principal investigator.

Chapter 4.1:

Lerner-Ellis J.P: Writing, data analyses, genotyping, DNA extraction, candidate gene selection, primer design, PCR, sequencing and analyses, mutation annotation, comparative genomics and functional sequence searches, cross species comparison of *MMACHC,* cloning, genotype-phenotype correlations, sequence alignment of TonB and molecular modeling, haplotype analysis, interpretation of the data and hypothesis on functional aspects of the gene product.

Tirone J.C: Initiation of the homozygosity mapping and sibling pair analysis project; DNA extraction, genotyping, candidate gene search, primer design and PCR.

Pawelek P.D: Comparative sequence analysis & molecular modeling.

Doré C: PCR of candidate genes, sequencing and analysis.

Atkinson J.L: Linkage and haplotype analyses.

Watkins D: Complementation and biochemical studies of infected cell lines, data analysis, copy editing.

Morel C.F: Data organization and analyses, mutation annotation, genotype-phenotype correlations and ethnic specific observations, haplotype analysis.

Fujiwara T.M: Data analysis, organization and compiling of sequencing data, mutation and genotype analysis and annotation, haplotype analysis, pedigree design, copy editing.

Moras E: PCR.

Hosack A.R: Resource manager, project design, copy editing.

Dunbar G.V: Maintenance and growth of all cell lines.

Antonicka H: Construction of vector, packaging of *MMACHC* and infection of *cblC* and control cell lines.

Forgetta V: Evolutionary comparison by alignment of gDNA of multiple species, quality control of sequence data.

Dobson C.M: Analysis of EST databases, multiple sequence alignment, editing.

Leclerc D: Analysis and identification of functional domains, editing.

Gravel R.A: Collaborator.

Shoubridge E.A: Design of packaging and infection experiments, copy editing.

Coulton J.W: Confirmation and analysis of TonB domain, copy editing.

Lepage P: Quality control and troubleshooting for candidate gene sequencing, primer design, EST database analysis, mutation analysis.
Rommens J.M: Linkage study and analysis of candidate region, Northern blot analysis, copy editing.
Morgan K: Project design, approaches to gene identification: marker selection, homozygosity mapping, haplotype and sibling pair analyses; haplotype analysis of SNP data using Phase 2.1.1, copy editing, ethnic specific observations.
Rosenblatt D.S: Principle investigator.

Chapter 5.0

Morel C.F: Writing, data analysis, phenotype-genotype correlations.
Lerner-Ellis J.P: DNA extraction, PCR, sequencing and mutation analysis, copy editing.
Rosenblatt D.S: Principal investigator.

ABBREVIATIONS

AdoHcy: S-adenosyl homocysteine

AdoMet: S-adenosylmethionine. MCM: Methylmalonyl CoA mutase. MS: Methionine Synthase

ATP: Adenosyl triphosphate

ATR: Adenosyltransferase

Cbl: Cobalamin

cDNA: complementary DNA

CRM: Cross reactive material

DNA: deoxyribose nucleic acid

GADPH: Glyceraldehyde-3-phosphate dehydrogenase

gDNA: genomic DNA

GDP: Guanine di-phosphate

GIF: Gastric intrinsic factor

GMPPNP: 5'-guanylylimidodiphosphate

GSH: Glutathione

GTP: Guanine tri-phosphate

HC: Haptocorrin

IF: Intrinsic factor

kcat: Overall catalytic constant

MMA: methylmalonic aciduria

MMAA: Gene name for methylmalonic aciduria cblA type

MMAB: Gene name for methylmalonic aciduria cblB type

MMACHC: Gene name for methylmalonic aciduria and homocystinuria cblC type

MTHFR: methylenetetrahydrofolate reductase

OHCbl: hydroxocobalamin

PAGE: Polyacrylamide gel electrophoresis

RNA: ribonucleotide tri-phosphate

RT-PCR: reverse transcription-polymerase chain reaction

SCD: subacute combined degeneration of the spinal cord

SDS: Sodium dodecyl sulphate

SNP: Single nucleotide polymorphism

TC: Transcobalamin

TCI: Transcobalamin I

TCII: Transcobalamin II

TCIII: Transcobalamin III

TDT: Transmission disequilibrium test

THF: Tetrahydrofolate

TLC: Thin layer chromatography

TABLE OF CONTENTS

TABLES & FIGURES

Introduction

Chapter 2

Chapter 3

Chapter 4

Chapter 5

CHAPTER 1.0: INTRODUCTION

1.0 Rationale and Objective of Research:

Over the past three decades, patients with inborn errors of vitamin B_{12} (cobalamin; Cbl) metabolism have been separated into eight different classes (*cblA-cblH*) on the basis of complementation analysis (1). Our objective has been to identify the genetic bases of disease for the different complementation groups. The primary focus of this thesis is the identification and characterization of the gene responsible for the *cblC* class. In addition, an objective of this research has been to characterize the spectrum of mutations in two recently identified genes responsible for the *cblA* and *cblB* inborn errors of cobalamin metabolism and to study the biochemical behaviour of patient cell lines in culture.

1.1 Milestones of Vitamin B_{12}

The discovery and purification of vitamin B_{12} has been described as a "major medical triumph of the first half of the 20^{th} century" (2). In fact, the elucidation of the three dimensional structure of vitamin B_{12} in 1956 by Dorothy Hodgkin and co-workers was a milestone not only for vitamin B_{12} research but also for crystallography, which has since proven to be an indispensable tool (3,4). Since Hodgkin's crystallography work, at least twenty-six different crystal structures of vitamin B_{12} have been determined, extensive work and novel mechanisms in the chemistry, biochemistry and biosynthesis of vitamin B_{12} and affiliating enzymes has been documented and at least fifteen different hereditary human diseases of absorption, transport and cellular vitamin B_{12} processing have been identified. In the latter half of the 20^{th} century and the beginning of the 21^{st}, recombinant DNA technology and the sequencing of genomes from multiple organisms, inaugurated a new era in the study and in our understanding of the role of this molecule in nature. The identification of the genes that, when mutated, cause inherited defects of vitamin B_{12} metabolism has provided insight into the biological function of this vitamin. The study of nutritional behavior in bacterial mutants has been critical to the genetic characterization of vitamin B_{12} pathways in bacterial systems (5) and to the genetic identification and characterization of the vitamin B_{12} pathways in the human diseases (1).

1.2 Chemical Structure of Cobalamin

Vitamin B_{12} (Cobalamin; Cbl) is a complex organometalic compound that consists of three parts: a planar corrin ring (biosynthetically and structurally related to those of heme and chlorophyll) with a central cobalt atom, a phosphoribo-5,6-dimethylbenzimidazolyl group that is coordinated to the α-axial position of the cobalt, and at the ß-axial position several different chemical moieties can bind (Figure 1). The composition of vitamin B_{12} is $C_{63}H_{88}O_{14}N_{14}Pco$ and the molecular weight of AdoCbl is 1580 Da (3). The dimethylbenzimidazole group is covalently attached to the corrin ring as part of a nucleotide loop. It is linked through its phosphate to aminopropanol, and aminopropanol is attached to a propionyl group that extends from the D porphyrin of the corrin ring.

The central cobalt atom exists in three different oxidation states including the oxidized trivalent state (cob(III)alamin), divalent (cob(II)alamin) and the fully reduced monovalent state (cob(I)alamin), and must be in the monovalent state before other chemical moieties can bind to the ß-axial position.

Only three Cbls have been routinely isolated from mammalian tissues: hydroxocobalamin (OHCbl), methylcobalamin (MeCbl) and adenosylcobalamin (AdoCbl). Cyanocobalamin (CNCbl) is a nonphysiological stable form of the vitamin that is used commercially and does not occur naturally; it is an artifact of purification. Glutathionyl- (GSCbl) and sulfito- forms of cobalamin have also been reported (6). MeCbl and AdoCbl are unique in that they are the only two naturally occurring compounds with carbon-cobalt bonds. AdoCbl has a 5'-deoxyadenosyl moiety serving as the β-ligand that is joined to the cobalt within the corrin ring by the 5'carbon of adenosyl. Similarly, the methyl group of MeCbl is covalently bound as the β-ligand, forming a carbon-cobalt bond.

MeCbl and AdoCbl are also unique in that they act as specific cofactors for two mammalian enzymes, methionine synthase and methylmalonyl-CoA mutase, respectively.

Figure 1

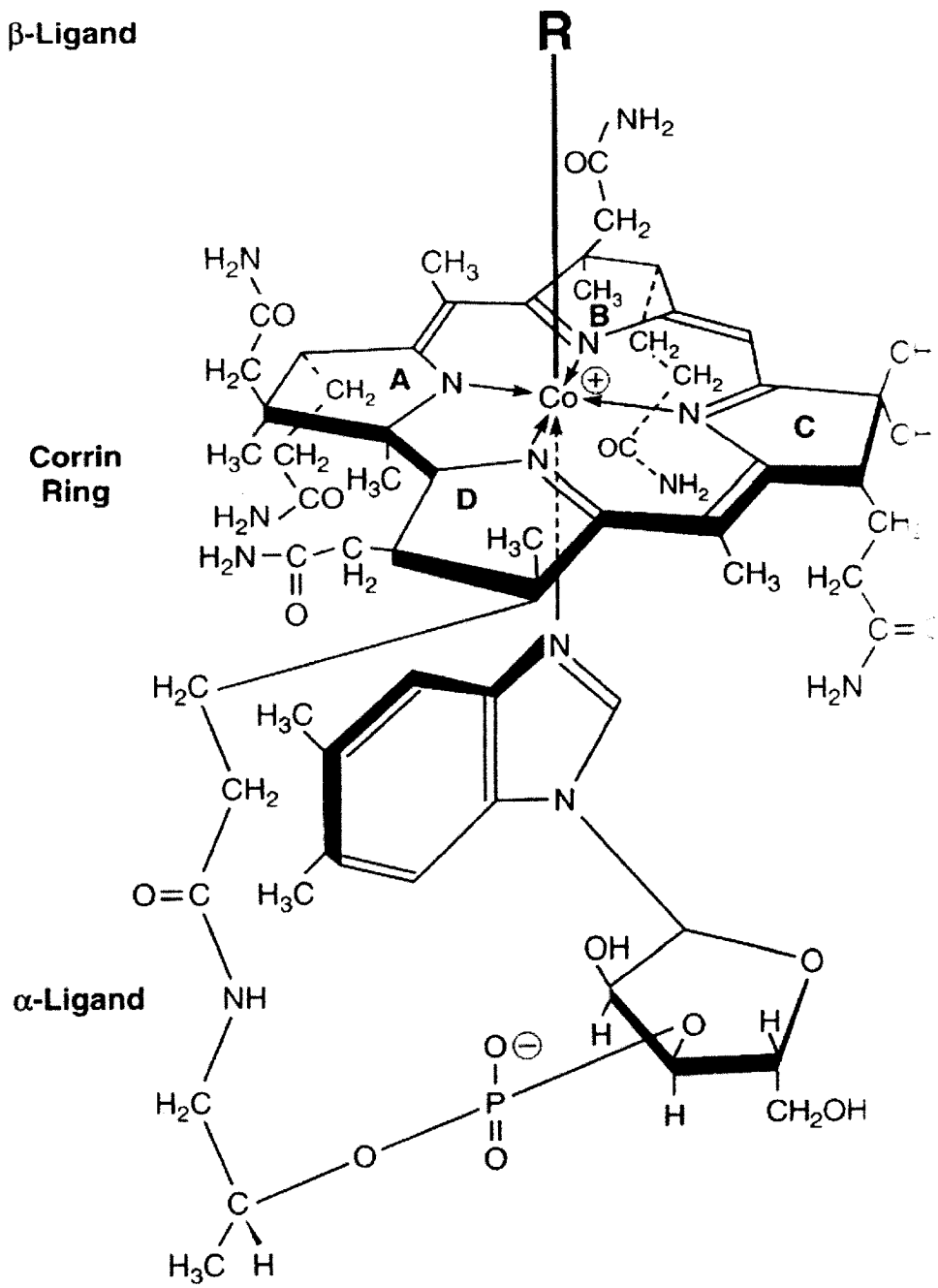


Figure 1: Chemical structure of Vitamin B_{12} / Cobalamin. Reprinted from Babior BM. Cobamides as cofactors: Adenosylcobamide dependent reactions, in: Cobalamin Biochemistry and Pathophsiology. New York, Wiley, 1975, p.141. ISBN:0471039705.

1.3 Origins & Evolution of Cobalamin

1.3.1 Distribution among life forms

Cobalamin is unevenly distributed among life forms, raising questions about its origins and biological significance. Cobalamin synthesis is restricted to certain bacteria and archaea; it is not involved in the metabolism of most plants, fungi or some bacteria. An aquatic fungus, *Blastocladiella emersonii*, was recently demonstrated to have retained cDNAs encoding enzymes from a coenzyme B_{12}-dependent pathway, suggesting that ancestral characteristics of prokaryotes may have been retained in this lineage at the base of the fungal phylogenetic tree (7). Genetic studies in bacteria have led to the discovery of at least 25 bacterial enzymes uniquely involved in vitamin B_{12} synthesis and two distinct pathways have been identified, one requiring molecular oxygen and the other an anaerobic process (8,9,5). Cobalamin is essential to eukaryotes because they are not able to synthesis cobalamin *de novo*. Therefore, humans and all mammals require dietary intake of cobalamin for survival.

1.3.2 Origins of Cobalamin

It has been suggested that cobalamin was important in the "RNA world" (10). Porphyrins have been synthesized non-enzymatically in primitive earth experiments and the complex vitamin B_{12} structure can be formed by energetically favorable reactions (11,12,13). Structural considerations of uroporphyrinogen III, the common precursor of heme, siroheme, chlorophyll, and cobalamin (13) have suggested that the cobalamin, heme, chlorophyll, and siroheme biosynthetic pathways may reflect the evolution of energy metabolism and that this pathway first evolved to produce cobalamin. It is believed that cobalamin was initially used to support anaerobic fermentation of small molecules by generating internal electron sinks. Later, siroheme evolved and allowed inorganic molecules to be used as electron acceptors followed by chlorophyll and heme that allowed for biological formation of molecular oxygen and use of oxygen as a respiratory electron acceptor (5).

1.3.3 Cobalamin utilization in bacteria

The original significance of cobalamin and its remaining primary role in modern bacteria is thought to be to support fermentation of small molecules by catalyzing molecular rearrangements that generate both an oxidizable compound and an electron sink for use in balancing redox reactions. Enteric bacteria carry out this role by cobalamin-dependent degradation of ethanolamine, propanediol, and glycerol. Both aerobic and anaerobic Cbl biosynthesis pathways have been identified in bacteria (14). The loss of Cbl synthesis enzymes from different organisms has helped us understand the evolutionary properties of Cbl-catalyzed reactions. The earliest use of Cbl may have been to support anaerobic fermentative growth at the expense of small molecules. Methionine synthesis and nucleotide reduction appeared as secondary uses. When oxygen and aerobic respiration first appeared on earth, many organisms no longer needed to perform fermentations and lost some of their original enzymatic capabilities while retaining a continued requirement for Cbl such as in methyl transfer reactions. Obligate aerobes and animals require Cbl to perform these non-fermentative functions (5). In nonenteric bacteria, the Cbl-dependent amino mutases catalyze mechanistically similar reactions that support fermentation of the amino acids (glutamic acid, lysine, leucine, or ornithine) and in methanogens, Cbl acts as a carrier of methane (15).

1.4 Isolation and Discovery of Coenzyme Activity

1.4.1 Isolation of vitamin B_{12}

In 1948, Folkers and coworkers isolated a red crystalline compound from liver. The factor was called vitamin B_{12} (16,17). Smith and Parker simultaneously reported purification of anti pernicious anemia factor, which was present in beef extract, milk powder, and culture broths of multiple bacterial strains. This was followed by the isolation and determination of the electronic spectrum of the crystalline anti-pernicious anemia factor from liver by Ellis *et al* in 1949 (18). In the years that followed additional structural and chemical details of vitamin B_{12} were unraveled revealing a compound of

"frightening complexity" and finally in 1956 Hodgkin elucidated a more complete structure of vitamin B_{12} by X-ray crystallography (3,4).

1.4.2 Cobalamin - a Cofactor for two Enzymes

In 1955, Flavin *et al* were among the first to discover the methylmalonyl CoA mutase (MCM) reaction (19) and that a vitamin B_{12} coenzyme is required for this reaction (20). Also among the first evidence that vitamin B_{12} was required as a cofactor came with the discovery by Barker *et al* (21) when he reported the isolation of a charcoal absorbable substance that was a cofactor for glutamate mutase. This yellow-orange cofactor was found to be sensitive to light. He went on to demonstrate that glutamate mutase, from the anaerobic bacterium *Clostridium tetanomorphum*, specifically required AdoCbl in the conversion of L-glutamate to threo-ß-methyl-L-aspartate (21). Shortly thereafter, the analogous isomerization of methylmalonyl CoA to succinyl CoA was demonstrated to be absent in the liver of Cbl-deficient rats (22) and the *in vitro* activity of methylmalonyl CoA mutase could be restored to normal by addition of AdoCbl but not by other cobalamin analogues (23,24). Later, Guest *et al* (25) would show that methylcobalamin was a substrate for methionine synthase (MS) and an important cofactor in the methylation of homocysteine to form methionine; the reaction requires N5-methyltetrahydrofolate and S-adenosylmethionine (AdoMet) (26,27).

Since these initial discoveries the mechanism of AdoCbl-dependent MCM activity and MeCbl-dependent MS activity have become better understood. MCM kinetic and isotope studies have provided insight into the rate of reaction and the 1,2 interchange between an acyl CoA substituent and a hydrogen atom on vicinal carbons (28,29). MS studies have shown the involvement of four binding domains including those for 5'-methyltetrahydrofolate, MeCbl, S-adenosylmethionine and homocysteine. More recently, crystal structures and spectroscopic studies have led to the understanding of cofactor binding and activity (30). Since the original identification of MCM and MS, 11 cobalamin-dependent enzymes have been identified: four mutases, four eliminases, and three aminomutases (8).

1.5 Biological importance of cobalamin in mammals

Two enzymes in mammals require cobalamin as cofactors: methylmalonyl CoA mutase (EC 5.4.99.2) utilizes AdoCbl, and methionine synthase (EC 2.1.1.13) utilizes MeCbl. Methylmalonyl CoA mutase converts L-methylmalonayl CoA to succinyl CoA and methionine synthase converts homocysteine to methionine. The biochemical abnormalities in patients with Cbl deficiency reflect the dysfunction of the enymes dependent on Cbl: methylmalonic aciduria and homocystinuria or both in patients with a defect in MCM or MS function or both.

1.5.1 Methionine Metabolism:

Methionine is an essential amino acid in mammals, required for protein synthesis and one carbon metabolism. Methionine is demethylated by various S-adenosylmethionine-dependent methyltransferase reactions to produce homocysteine, and two enzymes remethylate homocysteine. The cobalamin-dependent enzyme methionine synthase is responsible for maintaining adequate methionine levels and preventing accumulation of toxic homocysteine. A second cobalamin-independent enzyme, betaine:homocysteine methyltransferase (EC 2.1.1.5), is present in liver (31); however, this enzyme is insufficient to prevent homocysteine accumulation and its sequelae in cases where cobalamin-dependent methionine synthesis is defective. Homocysteine may also react with serine to form cystathionine in the reaction catalyzed by the enzyme cystathionine β-synthase (EC 4.2.1.22).

The activated form of methionine, AdoMet, synthesized by methionine adenosyltransferase (EC 2.5.1.6), is a methyl donor in at least 39 cellular transmethylation reactions including DNA methyltransferases 1 and 3, which catalyze methylation of cytosine residues at CpG sites of DNA, as well as enzymes catalyzing methylation of RNA, lipids, proteins (including histones), neurotransmitters and thiols (32). S-adenosyl homocysteine (AdoHcy) is a byproduct of these reactions and is hydrolyzed to homocysteine by the enzyme S-adenosylhomocysteine hydrolase (EC 3.3.1.1). However, AdoHcy hydrolase favors synthesis rather than hydrolysis of AdoHcy (33) and accumulation of AdoHcy can have adverse effects on cellular function because AdoHcy

inhibits various AdoMet dependent methyltransferases (32). Accumulation of intracellular homocysteine results in increased levels of AdoHcy.

While proper methionine homeostasis is required for normal DNA synthesis and other essential processes, the MCM branch of the cobalamin pathway is essential for normal catabolism of some branched chain amino acids and odd chain fatty acids. Amino acids such as isoleucine, valine, methionine, and threonine and fats such as cholesterol and odd-chain fatty acids are all catabolized generating L-methylmalonyl CoA which is metabolized by MCM, and deficient MCM activity leads to accumulation of the acid form of methylmalonyl CoA in blood and urine and causes metabolic acidosis in affected individuals.

Cobalamin plays an important role in DNA synthesis, and the haematological and neurological symptoms seen in pernicious anemia or inborn errors of cobalamin metabolism reflect the underlying enzymes involved in cobalamin metabolism. To date, at least eight intracellular enzymes are known to be defective in patients with inborn errors of cobalamin metabolism. The key step that emphasizes the involvement of cobalamin in DNA synthesis is its role as a cofactor for the cytoplasmic enzyme methionine synthase. Cobalamin accepts a methyl group donated from 5-methyltetrahydrofolate (5-methylTHF) to form MeCbl. This reaction occurs after binding of cobalamin (cob(II)alamin or cob(I)alamin) to methionine synthase. MeCbl acts as an intermediate methyl carrier between 5-methylTHF and homocysteine. The methyl group is subsequently donated to homocysteine to regenerate methionine. 5-methylTHF monoglutamate is the circulating form of folate and methionine synthase is the only enzyme that uses it, and thus regenerates THF. Because methylenetetrahydrofolate reductase (MTHFR) (EC 1.5.1.20) catalyzes the irreversible conversion of 5,10-methyleneTHF to 5-methylTHF in the cell, folate entering cells must pass through the methionine synthase reaction to generate tetrahydrofolate and the other folate derivatives which are involved in purine and pyrimidine metabolism (34,35,36).

In diseases affecting the processing of cobalamin and in cases of cobalamin deficiency, methionine synthase activity may be absent or decreased resulting in accumulation of 5-methylTHF and homocysteine and reduced levels of methionine and S-adenosylmethionine. S-adenosylmethionine normally functions as an inhibitor of

MTHFR. The result is that the MTHFR reaction proceeds with circulating levels of folate being trapped as 5-methylTHF, the basis of the "folate trap" hypothesis. Folic acid and folinic acid can be used clinically to bypass this block (1). Most of the 5-methylTHF in the cell remains in the monoglutamate form, and 5-methylTHF monoglutamate is a poor substrate for folylpolyglutamate synthase, the enzyme that converts folate monoglutamates to polyglutamates, which are normally maintained as the storage form of folate in the cell and are the only forms likely to be active with folate-dependent enzymes at physiological folate concentrations. Polyglutamates are not only retained better in cells, they are better substrates for most folate-dependent reactions, and in multifunctional enzymes the polyglutamate tails allow 'channeling' of folate between active sites (1).

1.5.2 Dietary sources and requirements

Humans do not synthesize cobalamin *de novo* and are dependent on dietary sources of the vitamin. Cobalamin is obtained from animal sources including foods prepared using bacterial cultures such as yogourt or cheese.

The recommended daily allowance of cobalamin for adults is 2.4 μg/day, and assuming ~50% absorption of at least 1 μg/day (37). The World Health Organization recommends daily intake of 1 μg for normal adults; 1.3 and 1.4 μg for lactating and pregnant women, respectively; and 0.1 μg/day for infants. These values are based on the known physiology and turnover of cobalamin and other factors.

Inadequate intake of cobalamin causes deficiency. Diets in developed countries generally contain adequate amounts of cobalamin. Deficiency may occur in vegans, lactovegetarians and in nonvegetarians whose diets contain little animal products (38,39,41).

1.6 Inherited Metabolic Disease of Cobalamin – Presentations & Characterization of Disease Groups

1.6.1 Clinical Observations

Rosenberg and Lindblad and co-workers were among the first to report children with a defect in the proper synthesis of the cobalamin coenzyme AdoCbl that resulted in

impaired mutase activity (42,43,44). These children had methylmalonic aciduria that responded to pharmacologic amounts of CNCbl or AdoCbl. Prior to this (in 1967) Oberholzer and Stokke (45,46) had described infants with metabolic ketoacidosis with methylmalonic aciduria who did not respond to Cbl supplementation signifying a primary block in methylmalonyl CoA mutase activity. In other words, patients who had methylmalonic aciduria could be divided into two groups: those individuals who responded to administration of pharmacologic doses of Cbl, and those children who failed to respond to Cbl supplements. Morrow *et al* (47) demonstrated evidence for biochemical heterogeneity among the methylmalonic acidemias. In four patients they measured the conversion of DL[^{3}H]methylmalonyl CoA to [^{3}H]succinyl CoA in liver homogenates and showed that three patients had no detectable response when AdoCbl was added to saturate the normal enzyme whereas in the fourth, mutase activity was restored to control values by AdoCbl. Evidence of additional defects in Cbl metabolism came in 1969 and 1970, when Mudd, Goodman and their colleagues described children who, in addition to having methylmalonic aciduria, had homocystinuria, cystathioninuria and hypomethioninemia; this was evidence for defective synthesis of both Ado- and Me-Cbl coenzymes (48,49). The identification and classification of isolated forms of homocystinuria followed only in the mid 1980's when complementation studies were developed and biochemical heterogeneity in this group was also identified (50,51,52,53).

1.6.2 Cell culture studies: genetic heterogeneity

Early studies of human fibroblasts demonstrated biochemical heterogeneity among individuals with cobalamin-responsive methylmalonic aciduria by measuring the ability of intact fibroblasts to synthesize AdoCbl and MeCbl from hydroxo-[^{57}Co]cobalamin (OHCbl) added to the growth medium. Two groups were observed, *cblA* and *cblB*, that failed to synthesize AdoCbl in intact fibroblasts but had normal MeCbl synthesis. Examination of crude cell extracts (in the presence of reducing agent) demonstrated that *cblA* extracts had normal synthesis of AdoCbl and *cblB* extracts did not, while both classes lacked methylmalonyl-CoA mutase activity in intact cells.

A third mutant class, *cblC*, did not produce AdoCbl or MeCbl in intact cells but had normal synthesis of AdoCbl in crude extracts; these mutants lacked MCM and MS activities in intact cells (these mutants have entirely normal specific activity of both MCM and MS, but the enzymes do not function in intact cells because of deficiency of the cobalamin cofactors) (54,55). These findings were reinforced when Gravel *et al.* in 1975 demonstrated genetic complementation in heterokaryons of human fibroblasts defective in cobalamin metabolism when fused by exposure to Sendai-virus. In this way, cell lines with defective Cbl metabolism were demonstrated to comprise three different groups (*cblA*, *cblB*, and *cblC*) and a fourth group corresponded to a primary defect in the mutase apoenzyme (*mut*) (56). It was further demonstrated that the mut group could be subdivided into mut^0 and mut^-; mut^0 patients having no mutase activity in the presence of cobalamin in cell culture and mut^- having some mutase activity under these assay conditions; mut^0 had no cross reactivity with antibody whereas mut^- did (57,58).

Complementation analysis demonstrated that a patient (one of two brothers, one asymptomatic) who had been described in 1970 with a clinical presentation very similar to *cblC* in fact belonged to a new complementation class designated *cblD* (49,59).

In 1986 a patient was described with methylmalonic aciduria with an inability to release free cobalamin from lysosomes. This patient's cell lines complemented cell lines from all known inborn errors of cobalamin metabolism and was designated *cblF* (60,61,62,63).

In 1984 a child was reported with isolated homocystinuria and disease in this individual was designated as belonging to a novel complementation group called *cblE* (51,64). By the time complementation analysis was performed on cells from this patient, several additional patients were identified with isolated homocystinuria and similar clinical findings. Analysis of fibroblast cells from these patients demonstrated normal cobalamin uptake but decreased synthesis of MeCbl, and decreased intact cell methionine biosynthesis as measured by incorporation of label from 5-[^{14}C]methyltetrahydrofolate into acid-precipitable material compared with control fibroblasts. Synthesis of AdoCbl and MCM function were normal. However, these cell lines could be further differentiated by analysis of MS activity in fibroblast extracts under standard assay conditions, with reducing agent (150μM 2-mercaptoethanol) and with suboptimal concentrations of

reducing agent. Some cell lines, including the index case with *cblE*, had normal MS activity in the presence of optimal reducing agent, with less activity than controls when reducing agent concentration was reduced; the other group had decreased activity under all conditions tested. This was evidence for genetic heterogeneity which was demonstrated further by showing that these patients fall into two different complementation groups: *cblE* and *cblG*. To this day complementation analysis serves as an important tool in the diagnosis of patient cell lines and classification of disease groups (64).

Recently, a patient with isolated methylmalonic aciduria whose cells complemented all other inborn errors of cobalamin metabolism that were tested, was classified as *cblH* (65). Complementation against a *cblD* cell line was excluded from this study with reasonable doubt of its involvement in isolated methylmalonic aciduria (*cblD* had already been classified as a disease of combined methylmalonic aciduria and homocystinuria). The recent identification of patients whose cells belong to the *cblD* complementation group, but who have isolated methylmalonic aciduria (*cblD variant 2*) or isolated homocystinuria (*cblD variant 1*) or both (*cblD*), raised the possibility that the *cblH* cell line may fall into the same group as *cblD variant 2* – an unusual example of clinical and biochemical heterogeneity at one genetic locus (66).

1.7 Cobalamin absorption and transport

Cbls have a specialized mechanism of intestinal absorption combining gastric, ileal and pancreatic components. Three proteins are known to be involved in uptake and transport of cobalamin: intrinsic factor (IF); transcobalamin (TC; TCII); and haptocorrin (HC; TCI; TCIII; R binder). These three proteins are similar in primary sequence and intron/exon structure and have been suggested to have arisen by duplications of an ancestral gene (67,68). They have 33% homology and specific domains have 60-80% sequence identity (69).

In humans, the gastric intrinsic factor (GIF) gene on chromosome 11q13 encodes IF (70). Intrinsic factor is synthesized by gastric parietal cells, producing a glycoprotein of 45 kDa that is required for uptake of dietary cobalamin in the intestine.

Several events precede cobalamin binding to IF (71,72). Cbls are first released from dietary protein in the acidic environment of the stomach followed by binding to HCs which are salivary and gastric proteins with high affinity for Cbl. TCN1 is located on chromosome 11q11-12 and encodes the haptocorrins, proteins of ~46 kDa that differ in their degree of glycosylation (73). The haptocorrins are synthesized by myeloid cells and are present in secretions including plasma, bile, saliva, tears, breast milk, amniotic fluid, and seminal fluid, and in extracts of granulocytes, salivary gland, platelets, hepatoma cells, and breast tumors (74,75,76).

Pancreatic proteases then break up the HC-cobalamin complex and liberate Cbls in the duodenum where they are able to complex with IF. Subsequently, the IF-Cbl complex interacts through its protein moiety with a specific ileal receptor composed of two proteins: one is called cubilin (*CUBN* gene on chromosome 10p12.1) and the other is called amnionless (*AMN* gene on chromosome 14q32). Cubilin is a 460 kDa protein located on the brush border of ileal cells, the renal proximal tubule and yolk sac. It consists of a 113-residue N-terminal region, eight epidermal growth factor-like repeats and 27 110-amino acid CUB domains (77). Amnionless is ~48 kDa and expressed in the same tissues as CUBN. Cubulin and amnionless form a complex known as "cubam" which is required for normal uptake of intrinsic factor-cobalamin (78,79,80,81). The IF-Cbl binds cubam and is transported into the enterocyte by an endocytic mechanism. The complex is dissociated and Cbl is transported in the cell across the basal membrane and into the portal blood, bound to transcobalamin, the transport protein for newly absorbed vitamin (82). TC is encoded by the TCN2 gene on chromosome 22q12-13 (68) and is a nonglycosylated protein of 43 kDa (83). It is found in plasma with a high turnover rate (84), cerebrospinal fluid, transudates, seminal fluid and is synthesized in numerous cell types including fibroblasts, macrophages, enterocytes, renal cells, hepatocytes, spleen, heart, gastric mucosa and endothelium (74). The three dimensional structure of TC has been determined and cobalamin shown to bind in the "base on" conformation between the two N-terminal α_6-α_6 barrels. The protein also has a small C-terminal domain (85). Based on studies of isolated domains from IF, a similar binding mechanism for cobalamin is expected for IF and HC (86,87).

Apical-to-basal transcytosis has been suggested based on studies of adenocarcinoma cells (88). However, it is currently not known exactly how Cbl is processed before release into the portal circulation and there is some suggestion that free Cbl must cross the lysosomal membrane before it can be released into the cytoplasm for metabolism and activation based on studies with chemicals that impair lysosome function (89) and studies on *cblF* patients who have impairment of cobalamin uptake from the intestine and whose cells accumulate free cobalamin in the lysosome due to a defect in the mechanism leading to Cbl exit into the cytoplasm (62). The IF-Cbl is rapidly internalized by endocytosis (90). Cbl is released from IF and IF is subsequently degraded. Cbl is believed to bind TC within the enterocytes and is released from the basolateral membrane; it is found in portal circulation bound to TC after approximately four hours (91,92,93,88).

The majority of plasma cobalamin is bound to haptocorrin (70-90%) in the form of MeCbl; the remainder is associated with transcobalamin. In addition, HC has considerable binding affinity for other corrins that lack vitamin B_{12} activity. It has been suggested that an important function of haptocorrins *in vivo* is the binding and excretion of such cobalamin analogues into the bile (94). The only Cbl available for uptake by cells other than hepatocytes is Cbl bound to TC. Cellular uptake is mediated by a specific unidentified receptor present on many cell types (95,82,96). TC-Cbl receptor has been purified but with inconsistent results between laboratories (97,98,99,100). TC-Cbl binds rapidly to the cell surface receptor followed by slow internalization then transcobalamin is degraded releasing free cobalamin (101,102). Endocytosis is believed to be the mechanism of TC-Cbl internalization and disruption of this process by preventing acidification of the lysosome with agents such as chloroquine or ammonium chloride has been demonstrated (103,104). A specific transport system is hypothesized to be required for transfer of Cbl to the cytoplasm. Patients with *cblF* disease accumulate free Cbl in the lysosome due to mutations in an as yet unidentified gene.

The maximum amount of IF-Cbl that can bind to receptors in the human intestine has been demonstrated to be ~ 1.5 μg. The body normally loses from 2 – 4 μg of Cbl per day. Cbl is excreted in the bile, binds intrinsic factor in the duodenum and can be

reabsorbed. Individuals with pernicious anemia do not reabsorp cobalamin thereby requiring regular intramuscular injections of Cbl (105).

1.8 Cellular cobalamin metabolism

The details of the cellular cobalamin pathway are only poorly understood but, with the identification of the genes involved and with the study of mutations associated with disease, these processes are being uncovered. Of the eight different defined complementation groups, seven of the genes responsible have now been identified, four in the last four years. The dissection of these genes and their functions has been of fundamental importance to our understanding of vitamin B_{12} biology. Figure 2 illustrates the details of the cellular cobalamin metabolism as it is currently understood and the following text describes the genes in the pathway, the method of gene discovery and what has been learned about their cellular functions.

The uptake of cobalamin in mammalian cells is mediated by specific receptors on the plasma membrane that bind the TC-cobalamin complex. Several studies have yielded ambiguous results as to the identity of the specific plasma membrane receptor (97,98,99). The identity and primary structure of the protein that mediates cellular cobalamin uptake remains unidentified. One report suggests that a 58kDa monomeric protein is the likely receptor for uptake of TC-Cbl (100). Cobalamin is transported into the cell by lysosomal mediated endocytosis bound to TC (106). Following degradation of TC in the lysosome, Cbl is released into the cytoplasm in the III^+ or II^+ oxidation sate, and must be reduced to its I^+ oxidation state before it can be adenosylated or methylated to form the active coenzyme forms. AdoCbl binds MCM in the mitochondria and is involved in converting L-methylmalonyl CoA to succinyl CoA, and MeCbl binds to methionine synthase in the cytoplasm where it participates in the catalysis of homocysteine to form methionine by donation of a methyl group.

Little is known about the mechanism of Cbl entry into the mitochondria. It is not clear whether Cbl enters in the Cbl II^+ or I^+ oxidation state but the current dogma suggests that it enters the mitochondria as Cbl II^+. It is also not clear whether Cbl is transported or diffuses into the mitochondria passively; both models have been suggested, although neither has been demonstrated. A study done on rat liver mitochondria demonstrated that

mitochondrial Cbl uptake is stimulated by mitochondrial swelling in the presence of Ca^{2+}(107). Several lines of evidence also indicate that the observed uptake of OHCbl involves processes in addition to diffusion across the mitochondrial membrane. (1) The inside/outside concentration ratio of OHCbl is much greater than the value expected if diffusion were the only process occurring. (2) The concentration dependence of OHCbl uptake is curvilinear, implying that a saturable process exists. (3) The uptake process demonstrates specificity. The mitochondrial uptake system is capable of distinguishing between cobalamins; CNCbl is taken up much less efficiently at all incubation times and concentrations than OHCbl and AdoCbl. (4) The uptake process involves the participation of a mitochondrial protein with a high affinity for OHCbl (107).

Figure 2

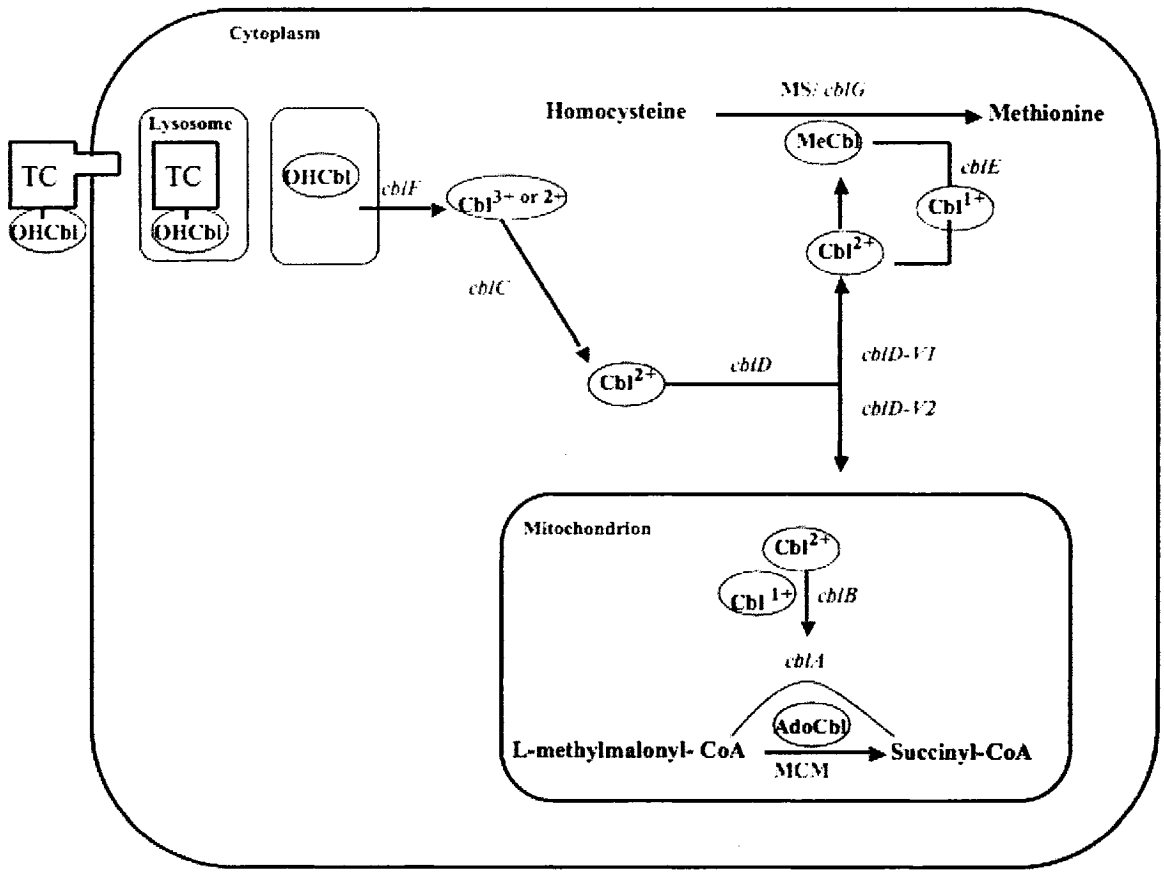


Figure 2. Cellular metabolism of cobalamin. TC: Transcobalamin; Cbl: Cobalamin; MS: Methionine synthase; MCM: Methylmalonyl-CoA mutase. The position of the inborn errors of cobalamin metabolism (cblA-cblG) are indicated as they are currently understood.

1.8.1 Molecular & Functional Aspects of Cobalamin Metabolism

cblF:

The genetic basis for *cblF* disease is not currently known. It is clear that these patients accumulate free CNCbl in the lysosome and that this block prevents synthesis of AdoCbl and MeCbl resulting in combined methylmalonic aciduria and homocystinuria. Presumably, patients with *cblF* disease have an inability to transport cobalamin out of the lysosome suggesting a possible defect in a lysosomal transmembrane protein (61,63,60).

cblC:

The *cblC* gene has long been hypothesized to have reductase or ß-ligand transferase activity (108) based on its apparent location in the metabolic pathway. In this thesis, we report the identification of the gene responsible for *cblC*, called *MMACHC*. The studies in chapters 4 and 5 examine details of the genetic and biochemical aspects of this gene and its product. Cobalamin binding and TonB-like domains were identified suggesting that MMACHC may be involved in the binding and trafficking of cobalamin inside the cell (see chapter 4 for details).

cblD:

Similar to *cblC* disease, *cblD* is due to an early block in vitamin B_{12} metabolism resulting in combined methylmalonic aciduria (MMA) and homocystinuria. However, this category has recently been subdivided into cases where either isolated MMA or isolated homocystinuria may occur (66). For this reason, the protein has been hypothesized to be multifunctional and may be involved in reducing and/or shuttling Cbl to the mitochondrial and cytoplasmic compartments. The gene responsible for this disease has not been reported.

cblG:

The methionine synthase gene was originally assigned to chromosome 1 based on hamster-human hybrid chromosomal transfer studies (109). In 1996 the human methionine synthase gene was cloned (localizes to 1q43 and called MTR, NM_000254) by using specific regions of homology to the known cobalamin binding domain within the methionine synthase sequences of *Escherichia coli* and by designing primers to amplify human cDNAs containing this sequence by RT-PCR (110). Others also cloned this gene independently (111,112). Confirmation of gene assignment to the *cblG* complementation group was based on identification of deleterious mutations in this gene in patients with *cblG* disease (110,113). A high degree of homology to the enzyme in other species was confirmed. The predicted sequence of the human enzyme is 55 percent identical to the cobalamin-dependent methionine synthase from *E. coli* (111). The three-dimensional structure of the cobalamin-binding and adenosylmethionine binding domains of methionine synthase from *E. coli* have been determined by X-ray crystallography (30,114) and the cobalamin-binding domain is homologous to the C-terminal cobalamin-binding motif of methylmalonyl CoA mutase. Crystal structures for the homocysteine-binding and methyl-THF-binding domains have also been determined from *Thermotoga maritime* (115) The human protein is a cytoplasmic enzyme of 1265 amino acids - 85-kDa - and functions as a monomer and contains a seven-residue structure-based sequence fingerprint for cobalamin binding. A polymorphism was identified (c.2756A>G (D919G)) which has implications in cardiovascular disease since reduced activity of methionine synthase can lead to elevated levels of homocysteine in the blood (110).

The function of methionine synthase, also known as 5-methyl-THF:L-homocysteine methyltransferase, has been well studied in bacteria. The enzyme has a number of binding sites including sites for cobalamin, folate, homocysteine, and S-adenosylmethionine. MS catalyzes the transfer of a methyl group from 5'methyl-THF (or adenosylmethionine) to homocysteine to form methionine, a process mediated by the cobalamin coenzyme. A methyl group from 5-methyl-THF is transferred to enzyme-bound cob(I)alamin to form methylcobalamin and the methyl group is then transferred to homocysteine producing methionine and regenerating cob(I)alamin. Enzyme-bound

cob(I)alamin is spontaneously oxidized after ~1000 cycles to cob(II)alamin, and is reduced by MTRR (a flavodoxin and NADPH reducing enzyme discussed below) and S-adenosylmethionine to regenerate methylcobalamin and for reactivation of the MS enzyme (116,117,118,119,120,121,122).

As mentioned previously, the cblG complementation group has reduced methionine synthase activity even under optimal assay conditions (53). However, biochemical heterogeneity has been noted (123). These findings suggested that some patients have a defect in catalytic domains of methionine synthase while others have a defect in the ability of Cbl to bind the apoenzyme (52,124). Mutations in the human MTR gene have been identified in cblG patients that disrupt the pocket where Cbl-binding occurs that normally accommodates the dimethylbenzimidazole moiety of the cofactor including I881delta and H920D (113,110). However, rather than being authentic Cbl binding mutants, some of the cblG variants appear to not express methionine synthase protein at all (125). Another more common missense mutation disrupts the adenosylmethionine binding site (P1173L) (113).

cblE:

Bacterial studies have shed much light on the processes of methionine synthase reductase function. Cleavage of the methyl-cobalt bond of methylcob(III)alamin intermediate occurs heterolytically as to leave the cobalamin in the highly reactive cob(I)alamin oxidation state. During the cycling between III^+ [i.e. MeCbl] and I^+ oxidation states, the cofactor may be spontaneously oxidized to cob(II)alamin with inactivation of the enzyme. Restoration of enzyme activity is dependent on the reductive methylation of cob(II)alamin in a reaction in which S-adenosylmethionine provides the methyl group (121,126). In bacteria, the reductive activation system consists of a two-component flavoprotein system, flavodoxin and NADPH-ferredoxin (flavodoxin) oxidoreductase (a member of the FNR family of electron transferases) (127,122). An NADPH-dependent reducing activity was shown to be defective in *cblE* patients suggesting that the reductive-activation system in higher organisms resembles *E. coli* (although it was known that the flavodoxin/flavodoxin reductase system does not exist in mammalian cells) (120). The

human counterpart was hypothesized to contain the FMN, FAD and NADPH binding motifs present in the two bacterial proteins in a single protein, since only a single complementation class was implicated. The gene responsible for the *cblE* complementation group was identified by using consensus sequences to predict binding sites for FMN, FAD, and NADPH. The gene *MTRR* was localized to chromosome 5p15.2-15.3. An mRNA of 3.6 kb was detected by Northern blot analysis and the protein is a novel member of the FNR family of electron transferases and contains 698 amino acids. The gene shares 38% identity (49% similarity) with human cytochrome P-450 reductase and 43% with the *C. elegans* putative methionine synthase reductase. The authenticity of the cDNA sequence was confirmed by PCR based techniques and gene assignment by identifying mutations in *cblE* patients (122).

mut:

Flavin *et al* (1955) and Katz & Chaikoff (1955) originally observed the isomerization of methylmalonyl CoA to succinyl CoA catalyzed by an enzyme found in sheep kidney and rat liver (19,128). Studies of a similar chemical reaction, the isomerization of glutamate to ß-methylaspartate in bacteria (21) and the demonstration by Barker and colleagues (20) that AdoCbl was required for this reaction led to the discovery that AdoCbl was also required for methylmalonyl CoA mutase activity (129,23,24). The enzyme has been purified from human, and crystallized from sheep kidney and bacteria (130,131). The human enzyme consists of two identical subunits (~ 77 kDa). The holoenzyme contains 1 mole of AdoCbl per mole of subunit (131), with Cbl being very tightly bound to the apoenzyme (K_m: 5 x 10^{-8} M) (132). It has been suggested that the active sites of the dimeric enzyme are not equivalent and that the enzyme displays complex kinetics with regard to methylmalonyl CoA and AdoCbl binding. OHCbl may act as a competitive and irreversible inhibitor of human mutase (133). AdoCbl plays a critical part in the intramolecular isomerization catalyzed by methylmalonyl CoA mutase. In this reaction, AdoCbl serves as a source of a free radical pair, by homolysis of the cobalt-carbon bond (134,135). The isomerization reaction likely occurs by exchange of a hydrogen for the CoA-carboxyl moiety of methylmalonyl CoA (136,137). Mutase from *Propionibacter*

shermanii has been purified, cloned, and overexpressed in *E. coli*. It consists of a αß heterodimer with a single active subunit (alpha) that binds one molecule of AdoCbl and is 60% identical to the human enzyme (138,139,140). Several forms of *P. shermanii* mutase have been crystallized identifying N-terminal subunit-subunit interaction and interdomain linker sites, TIM barrel with substrate binding site, Cbl-binding domain with a groove for binding 5,6-dimethylbenzimidazolyl side chain of Cbl with a domain interface that accommodates the upper face of the corrin ring and 5'-deoxyadenosyl group (141,142,143,144). Substrate-free and substrate-bound structures have helped to demonstrate binding of substrate, product release and conformational changes during these processes (144). The conformational change is initially triggered by substrate binding which drives homolysis of the cobalt-carbon bond while sequestering the radicals formed at the active site simultaneously. This permits a free-radical rearrangement protected from the surrounding aqueous environment.

The gene for human methylmalonyl CoA mutase (MUT) was identified and cloned by sequencing a liver cDNA library and identifying clones with sequence matching that of the known protein sequence, and localized using mapping techniques (cDNA from human/hamster somatic cell hybrids and *in situ* hybridization using liver cDNA library) to chromosome 6p12-p21.2 (145,146,147,148). Two types of apomutase defects are known and designated mut^0 and mut^-. Two thirds of mutase patients constitute the mut^0 group. Mutase activity in cell extracts is undetectable even in the presence of excess AdoCbl. Radioimmunoassay under steady-state conditions in cell lines from 21 patients demonstrated that 12 had no immunologically identifiable mutase protein whereas 9 had reduced amounts of cross reactive material (CRM) ranging from 1 to 40 percent of that found in control extracts (149). The second class, mut^-, involves a structurally abnormal mutase apoenzyme which retains anywhere from 2-75 percent of control activity and has a Km for AdoCbl ~ 200-5000 times normal, has a normal Km for methylmalonyl CoA, and exhibits increased thermolability relative to control enzyme (150,133,151). Immunologically reactive mutase in these extracts ranges from 20-100 percent of control (149). The differences between mut^0 and mut^- have been firmly established by the identification of nonsense and missense mutations at the MUT locus.

1.8.2 Prokaryotic gene arrangements and the identification of novel B_{12} genes: *cblA* and *cblB*

The genes responsible for *cblA* and *cblB* disorders have recently been identified (152,153) by analysis of bacterial gene arrangements. With the availability of the Clusters of Orthologous Sequence database, it has been possible to look for bacterial genes which share biochemical and DNA sequence similarity to human genes. The principles of prokaryotic gene arrangements for the identification of genes involved in cobalamin metabolism are as follows. In bacteria, genes involved in specific metabolic processes are grouped together in operons. By identifying genes in bacteria that are frequently arranged with methylmalonyl CoA mutase (*mcm*) in operons and inferring that conserved neighbors of *mcm* genes and their human orthologues were also involved in propionyl CoA or methylmalonyl CoA metabolism, these human orthologues could be screened by PCR and sequencing analysis for mutations in DNA from affected individuals with *cblA* or *cblB* disease.

In 2001, Bobik & Rasche identified the human methylmalonyl-CoA epimerase gene by analysis of prokaryotic gene arrangements (154). They showed that one *mcm* neighboring gene in *Pyrococcus horikoshii*, lactoylglutathione lyase (LGSH), showed sequence similarity to a region on human chromosome 2. They subsequently found that LGSH was misclassified and actually encodes a DL-methylmalonyl-CoA racemase (or epimerase). This was one of the first reports in which the function of a eukaryotic gene was determined using knowledge of bacterial gene arrangements. The correct gene assignment has more recently been confirmed by identification of a deleterious mutation in the *MCEE* gene in more than one individual having methylmalonic aciduria (155,156). In Bobik's initial report, two other neighboring genes were identified which were hypothesized to be involved in cobalamin metabolism. Human orthologues of these genes were subsequently screened for mutations by Dobson *et al* and were mutated in patients with the *cblA* and *cblB* disease groups (152,153). These genes were called *MMAA* and *MMAB* respectively, and their identification has led to numerous subsequent reports which have helped improve our knowledge of not only disease in *cblA* and *cblB* individuals but also of our understanding of cellular cobalamin utilization.

cblA: Functional and biochemical studies on *MMAA*

Early studies on patient fibroblast extracts suggested that *cblA* patients had a defect in a gene coding for a Cbl reductase or a mitochondrial transporter, given that extracts of *cblA* cells synthesized AdoCbl from exogenous CNCbl normally in the presence of a reducing agent (55). No transporter could be identified although the size of the cobalamin molecule might indicate that a transporter is necessary (157,158). One study suggested that an NADPH reductase involved in the reduction of $CblII^{+}$ to $CblI^{+}$ was the cause of MMA in one patient. This individual has recently been identified as having a mutation at the locus encoding *MMAA*, the gene responsible for the *cblA* disorder of Cbl metabolism, which does not have reductase activity (159,152).

The MMAA protein has a predicted mitochondrial leader sequence and signal cleavage site (P = 0.998; predotar: www.inra.fr/predotar) but does not contain domains suggestive of flavin or NADPH-binding sites expected in a cobalamin reductase. In considering a possible function for MMAA, the literature on ArgK was examined given that this gene in *E. coli* had 61% sequence similarity to MMAA. In *E. coli*, mutations in the argK gene result in a deficiency in the uptake of lysine, ornithine and arginine (LOA). The gene product was shown to have ATPase activity and phosphorylate the LAO and AO periplasmic binding proteins *in vitro*. These LAO/AO periplasmic binding proteins have been shown to be associated with two inner membrane transport systems, HisP and ArtP. Both are ATPases with ATP-binding cassette signatures. ArgK lacks an ATP-binding cassette signature, which challenges the notion that ArgK is involved in transport-associated ATPase activity (160,161,152). The involvement of ArgK in cobalamin transport was further investigated in *E. coli*. Phylogenetic analysis of nucleotide-binding proteins included ArgK as a member, proposing that it belongs to one of four subfamilies comprising the G3E family of P-loop GTPases. The G3E family has sequence signatures including Walker A and Walker B motifs, a Mg^{2+}-binding aspartate residue and a sequence specific to GTP binding. The G3E family also comprises three subfamilies represented by UreG, involved in assembly of metallocentre of nickel metalloenzymes; HypB, a hydrogenase expression protein involved in metal binding and metalloenzyme assembly; and CobW, a protein in the biosynthetic pathway of cobalamin whose role is undefined. There is no evidence that the ArgK protein plays a role in

cobalamin metabolism and study of the ArgK gene provides limited insight into the function of MMAA. Moreover, as mentioned above, the functional assignment of argK has been challenged (162,154,163,164).

Previous studies have suggested that cobalamin uptake occurred in isolated swollen rat liver mitochondria and was mediated by calcium or phosphate addition (165,166). This was hypothesized to occur through the mitochondrial permeability transition pore (MPTP) that is permeable to molecules of <1,500 Da, close to the molecular mass of cobalamin (1350 Da) (166). However, there is some question of how applicable these studies are to mitochondria under physiologic conditions. It has been speculated that MMAA might be involved in this process by stimulating ATP or GTPase activity to open the mitochondrial permeability transition pore (152). However, there is little to no evidence in support of this hypothesis.

Recent studies on the MMAA orthologue in *Methylbacterium extorquens*, MeaB, demonstrated that MeaB forms a complex with methylmalonyl-CoA mutase and stimulates *in vitro* mutase activity, which supports the hypothesis that MeaB may function to protect methylmalonyl-CoA mutase from irreversible inactivation. In several bacteria, MeaB is fused with MCM into a single polypeptide; genes that are fused into a single gene in bacteria are likely to encode polypeptides that interact in other organisms (167,168). Korotkova and Lidstrom created double mutants of the orthologous genes encoding MCM and methylmalonyl-CoA epimerase and MCM and cob(I)alamin adenosyltransferase which were able to grow on succinate but not on methanol and ethylamine unless glyoxylate was supplied in the medium. This phenotype was the same as observed in a MeaB mutant. No MCM activity was detected in any of these mutants in the absence of AdoCbl. When 50 μM AdoCbl was added to the reaction mixture, low MCM activity was detected in double mutants suggesting that MeaB might be necessary for preventing inactivation of MCM after catalysis by protection or reactivation function. Thus these double mutants support partial restoration of methylmalonyl-CoA mutase activity in the presence of exogenous AdoCbl. This result was interpreted as evidence that MeaB protects methylmalonyl-CoA mutase from suicide inactivation (167).

The MMAA gene was shown to have a GTPase/ATPase consensus-binding motif with a conserved aspartate residue characteristic of GTPases, suggesting the actual

substrate *in vivo* is GTP rather than ATP. Purified MeaB incubated with Mcm showed a time-dependent and $MgCl_2$-dependent decrease of GTP and formation of GDP by thin layer chromatography (TLC) suggesting that it binds GTP and catalyzes the hydrolysis of GTP to GDP. When MCM apoenzyme was incubated with MeaB, a new band appeared on non-denaturing PAGE. SDS-PAGE of the complex allowed detection of individual subunits, MCM and MeaB, indicating that these products form a complex. Addition of AdoCbl or CNCbl showed formation of a new major complex. Purified MeaB did not restore MCM activity in meaB mutant extract in the presence of AdoCbl and $MgCl_2$ with either GTP or GDP. This indicated that MeaB is not involved in release of cofactor and the catalytically inactive cobalamin species from MCM. However, MeaB was demonstrated to increase MCM activity *in vitro*. ApoMCM (plus AdoCbl) was incubated in the presence and absence of MeaB (1:1), a mixture of MeaB and GTP, or a mixture of MeaB and GDP for 10 min and assayed using HPLC. In the presence of MeaB, the activity of MCM, as measured by the conversion of methylmalonyl-CoA to succinyl-CoA, increased from 3.9 +/- 0.1 units/mg protein, compared to 1.2 +/- 0.1 units/mg protein without addition of MeaB. In the presence of GDP or GTP in the reaction mixture with addition of MeaB yielded similar results, 4.1 and 4.3 units/mg protein, respectively. In summary, these data demonstrated a role for MeaB by binding to MCM and either stabilizing the dimer form of the enzyme or protecting the cofactor from attack by oxygen, water and reactive radical intermediates (167).

Recent studies using dynamic light scattering and size exclusion chromatography are consistent with MeaB forming a homodimer with each unit ~35 kDa in size. Nucleotide binding was shown to elicit a conformational change that decreases the radius of MeaB and suggesting a more compact structure for the binary complex. MeaB was shown to form a stable complex with methylmalonyl-CoA mutase and was resolved on a native polyacrylamide gel. Complex formation was monitored in the presence of GMPPNP (a nonhydrolyzable analog of GTP and GDP) and MeaB:methylmalonyl-CoA mutase saturate at a ratio of ~1:1, consistent with the association of one dimer of MeaB per heterodimer of methylmalonyl-CoA mutase. Addition of 5 mM GTP had no effect on binding stoichiometry. MeaB exhibits similar affinities for GMPPNP and the presence of mutase enhances the affinity for the nucleotides in the apomutase and holomutase forms

(51-fold and 8-fold increase respectively). The dissociation constant for binding of methylmalonyl-CoA mutase and MeaB ranged from 34 to 524 nM at 20°C depending on the combination of nucleotide and the form of mutase used. Holomutase exhibited a 15-fold higher affinity for the GMPPNP-bound form of MeaB compared to the GDP-bound form wherease the apomutase form binds both forms with comparable affinity (169).

Assessment of the binding and folding energetic relationships suggest that MeaB bound to GMPPNP buries 8600 and 5360 $Å^2$ of surface area, respectively, which was postulated to reflect conformational changes that are coupled to binding. MeaB had modest intrinsic GTPase activity (k_{cat} of ~0.04 min −1 at 37°C). In the presence of apo- or holo-mutase, the GTPase activity was enhanced by two orders of magnitude suggesting that the mutase functions as a mild guanine nucleotide-activating protein. In the presence of mutase, MeaB would accumulate in the GDP-bound state, which has lower affinity for the MeaB/MCM complex. The MeaB/GDP form has a 15-fold lower affinity for the mutase. MeaB binds GMPPNP ~4-fold more tightly than GDP (this difference disappears in the presence of 300 mM KCl which is likely more physiologically relevant). Methylmalonyl-CoA mutase binds to the MeaB/GTP complex and stimulates the GTPase activity of MeaB by a factor of ~100. Apomutase does not discriminate between the GDP- and GMPPNP-bound forms of MeaB in terms of binding affinity. Together these data suggest that the GTPase activity is harnessed by the MeaB/MCM complex to carry out a specific role (169).

A homology model of MeaB was generated using the crystal structure for the signal sequence-binding protein from *Thermus aquaticus*. The predicted structure has a core of α/ß G-domain comprised of parallel ß-strands surrounded by α-helices. The N-terminal Walker A motif (GXXGXGK(S/T) sequence is predicted to function in positioning the triphosphate moiety of the bound nucleotide. The Walker B motif contains a conserved aspartate that was suggested to play a conserved structural role and the (N/T)KXD sequence confers specificity for guanine (164). The N- and C-terminal regions of MeaB were shown to be organized into discrete modules and may be involved in interactions with methylmalonyl-CoA mutase and/or dimer formation (169).

Structural reorganization of MeaB is predicted to accompany binding of AdoCbl cofactor to the apomutase. MeaB is involved in the binding of AdoCbl to

apomethylmalonyl-CoA mutase in a GTPase-dependent step and protects the radical intermediates formed during the reaction catalyzed by the mutase in a GTP-independent manner. It was hypothesized that MeaB functions to gate cofactor binding to the mutase in a GTP-dependent manner and subsequently shown by isothermal titration calorimetry that no heat was released in the presence of GMPPNP bound to the mutase-MeaB/GTP complex suggesting the necessity for GTP hydrolysis for cofactor binding to the apoenzyme (170,171).

cblB: Functional and biochemical studies on *MMAB*

Expression of the human adenosyltransferase (ATR) fusion proteins and analysis by SDS-PAGE demonstrated the presence of two proteins of 56 and 52 kDa, close to the predicted molecular mass of ATR with and without the mitochondrial leader sequence. Both bovine and human ATR fusion proteins were examined for ATR activity. The highest specific activity for bovine and human ATR in cell extracts was 85.7 nmol/min/mg and 98 nmol/min/mg, respectively. When ATP or cob(I)alamin was excluded from the assay mixture, no activity was detected. The human ATR cDNA was shown to complement an ATR-deficient bacterial mutant for AdoCbl-dependent growth on 1,2-propanediol. Western blots were performed on lysates from *cblB* mutant cell lines using antibody against recombinant human ATR. Human ATR was reduced or not detectable in *cblB* mutant human skin fibroblasts providing direct evidence that defects in ATR underlie *cblB* disease (172).

Additional studies showed that R186W (the most common mutation observed in *cblB* patients) and E193K were associated with absent protein by Western blot. Wild-type MMAB and four mutant proteins (R186W, R190H, E193K, R191W) were stably expressed at high level as GST-fusion proteins. Only R191W was enzymatically active. It had an elevated Km of 320 μM (vs 6.8 μM for wild type enzyme) for ATP and 60uM (vs 3.7 μM for cob(I)alamin, with a reduction in k_{cat} for both substrates). Three mutant proteins examined by circular dichroism spectroscopy - retained an α-helical structure. Immunofluorescence co-localization of MMAB and mitochondria was also demonstrated

and is consistent with early studies on patients with isolated methylmalonic aciduria belonging to the *cblB* disease group (173).

Corrinoid adenosyltransferases have evolved in bacteria to accommodate different specific enzyme systems. They are grouped into three families: CobA, EutT and PduO. CobA is an ATP:corrinoid adenosyltransferase and participates in Cbl biosynthesis by the adenosylation of an intermediate prior to adenosylation of Cbl, but it can also adenosylate cob(I)alamin directly (174). The three dimensional structure of CobA has been solved and has a unique nucleotide binding to the P-loop compared to other nucleotide hydrolases (175). The other two cob(I)alamin adenosyltransferases, EutT and PduO, do not contain a P-loop-nucleotide binding motif and their mechanisms of function are not known. EutT is involved in the Cbl-dependent degradation of ethanolamine in *Salmonella typhimurium*. *MMAB* has sequence similarity to PduO, an ATP:cob(I)alamin adenosyltransferase, integral to growth on 1,2-propanediol (176). PduO was proposed to be a bifunctional protein, acting as an adenosyltransferase as well as a reductase that might convert cob(II)alamin to cob(I)alamin (177,176).

Spectroscopic studies have demonstrated that ATR binds cobalamin in the "base-off" (with the alpha ligand not coordinated to the cobalt atom) conformation in both the substrate cob(II)alamin and product AdoCbl oxidation states. ATR is trimeric and binds various cobalamin derivatives with a moderate affinity. A four-coordinate cob(II)alamin is observed in the presence of ATP with unusual electron paramagnetic resonance and magnetic circular dichroism spectroscopic properties that are observed (178). ATR was suggested to bind to AdoCbl and then pass cobalamin to MCM via MMAA.

1.9 Clinical and Laboratory Features

1.9.1 Pattern of Inheritance

All cobalamin related inborn errors are inherited as autosomal-recessive traits. This is based on the fact that equal numbers of affected males and females are present in each group; there is no instance of vertical transmission from affected parent to affected child. Interclass heterokaryons formed between cell lines from different etiologic groups complement each other in cell culture whereas intraclass heterokaryons do not with the exception of interallelic complementation in the mut and *cblA* groups (1). With the

description of the underlying genes it is possible to directly demonstrate autosomal recessive inheritance.

1.9.2 Prevalence of Disease

It is currently not possible to define the prevalence of these disorders in the general population. Two newborn screening surveys, one in Massachusetts and one in Quebec, suggest that methylmalonic acidemia may occur in 1:48,000 or 1:61,000 infants respectively (179,180). It was suggested that because this study included infants 3-4 weeks of age and because it is known that many children with methylmalonic aciduria die in the first week of life, that the true prevalence of disease must be greater. Disease involving combined MMA and homocystinuria may be even more common given the high frequency of *cblC* cases; however, no disease causing mutation has yet been observed in over 400 alleles examined from control cohorts.

1.9.3 Clinical Findings of Isolated Methylmalonic Aciduria: *mut*, *cblA*, *cblB*

Defects that affect only AdoCbl biosynthesis or a primary defect in the methlymalonyl CoA mutase enzyme generally lead to methylmalonic aciduria (MMA) and metabolic ketoacidosis in the newborn. To date ~ 300 cases of isolated methylmalonic aciduria have been diagnosed. Another ~200 patients have presented with mild methylmalonic aciduria but could not be assigned to any complementation class. Most of the patients with isolated MMA are accounted for by mutase deficiency (*mut*), the most common of the methylmalonic acidurias, which comprises ~150 individuals (in our cohort). In our laboratory there have been approximately 45 *cblA* and 40 *cblB* diagnoses. The clinical findings in *cblA*, *cblB*, and *mut* patients with MMA are very similar. The most common signs and symptoms at onset are lethargy, failure to thrive, recurrent vomiting, dehydration, respiratory distress, and muscular hypotonia. MMA seems to be continuous in the absence of treatment. Metabolic acidosis and hyperammonemia are characteristic of acute metabolic decompensation, and are generally short-lived – either the pH is controlled and returns to normal, or failure to control pH leads to patient death; the acidosis only lasts a few hours or a few days in any case.

Developmental retardation, hepatomegaly or coma were less common clinical manifestations and may result in the absence of diagnosis and treatment. Patients in the mut^0 class generally presented earlier than those in the other groups. About 80% of children in the mut^0 class presented in the first week of life. There was greater variability in the age of onset in the other complementation groups (181).

1.9.4 Laboratory Findings of Isolated Methylmalonic Aciduria

The laboratory findings in affected patients include normal serum Cbl concentrations; in the majority of patients, metabolic acidosis with blood pH values as low as 6.9 and serum bicarbonate concentrations as low as 5 mEq/L have been observed. Ketonemia or ketonuria was routinely found in patients (~80%) with hyperammonemia being only slightly less common, occurring in 70% of affected patients. Leukopenia, thrombocytopenia and anemia were the only other manifestations that were noted in 50% or more of this group of patients. Hypoglycemia may also present in these patients (181). Several patients have been reported with methylmalonic aciduria with no symptoms. These individuals were presumed to have a "leaky" enzyme defect that did not compromise homeostasis (182). Individuals ascertained by newborn screening with MMA in the urine were reported with levels less than 1400 mmoles/mmole creatinine, had normal somatic and cognitive outcomes (183).

Normal children and adults generally excrete less than 5mg methylmalonate daily, whereas children with isolated methylmalonic aciduria have excreted from 240 to 5700 mg in a 24-h period. Plasma concentrations of methylmalonate, almost undetectable in normal subjects, have ranged from 2.6 to 34 mg/dl in patients with methylmalonic acidemia. MMA is not the only abnormal metabolite found in the body fluids of these patients. Propionate and some of its precursors (butanone) or metabolites (ß-hydroxypropionate and methylcitrate) as well as acetic acid also accumulate in blood and urine in smaller amounts (42,184,185). There is a clear relationship between protein or amino acid loading and MMA accumulation (42,43,45,46). Originally, early screening tests for these disorders measured the ability of intact peripheral blood leukocytes or cultured fibroblasts to oxidize [^{14}C]succinate to $^{14}CO_2$ (1). This procedure has been

replaced by the less cumbersome procedure of incorporation of label from [^{14}C]propionate into trichloroacetic acid-precipitable material, as well as [^{57}Co]CNCbl uptake and AdoCbl formation by intact fibroblasts and genetic complementation studies in cultured fibroblasts (186,52).

1.9.5 Clinical Findings of Isolated Homocystinuria: *cblE & cblG*

Deficiency in the synthesis of MeCbl or the enzyme methionine synthase results in failure to thrive, megaloblastic changes, and neurologic signs, usually with homocystinuria and is the least common form of cobalamin disease. Two complementation groups fall into the category of isolated homocystinuria: *cblE* and *cblG*. In our lab, 25 *cblE* patients and 37 *cblG* patients have been diagnosed. Patients with either *cblE* or *cblG* disease have similar clinical and biochemical features. Most present in the first few months of life with vomiting, poor feeding and lethargy, severe neurologic dysfunction including cerebral atrophy, hypotonia, seizures and developmental delay. Megaloblastic anemia and homocystinuria are generally present; hypomethioninemia is common. Vision abnormalities and skeletal abnormalities are less common. Serum cobalamin and folate are normal or elevated and methylmalonic aciduria is absent except in one *cblE* patient where MMA was transient. Long-term prognosis remains unknown. The first *cblE* patient identified was reported at 18 years old as thriving and only mildly developmentally delayed, with a sibling identified prenatally who was normal at 14 years with a slight speech impediment (187,188). The index *cblG* patient, although clinically well, had remained significantly retarded with major visual defects (189). One *cblG* patient presented in mid-20s with sudden loss of motor and cognitive abilities (190).

There are three levels at which the impact of disorders involving defective methionine synthase activity are felt: hematologic, short-term neurologic and long-term neurodevelopmental. The hematologic problems might reflect disturbed DNA synthesis, while the short-term neurologic symptoms are likely due to either acute toxic effects or aberrant neurotransmitter metabolism (191). Long-term developmental effects appear to be related to defects in myelination in the central nervous system. Abnormal CT scans are common when the test is performed, as well as apparent atrophy or hypoplasia of the

brain; delayed myelination was shown by MRI in only a couple of patients. A number of suggestions have been made regarding neurological disruption. It may be due to toxicity of methylTHF or homocysteine, classic folate trap hypothesis, or reduced methylation of proteins and neurotransmitters due to deficiency in S-adenosyl-methionine synthesis (191).

1.9.6 Clinical Findings in Combined Methylmalonic Aciduria & Homocystinuria

cblC disease is the most common inborn error of cobalamin metabolism comprising at least 300 known cases. Individuals with *cblC*, *cblD* or *cblF* have methylmalonic aciduria and homocystinuria due to an early block in cellular cobalamin metabolism. Patients with *cblC* disease present with a wide range of symptoms. This observation has been made, in part, due to the large number of patients with *cblC* disease. Most patients with *cblC* present in the first year of life and ~10% of cases present in adulthood (192). In contrast to the *cblC* group, the original *cblD* patient only came to medical attention later in life at 14 years old. Findings included moderate mental retardation with neuromuscular problems involving his lower extremities and no hematologic abnormalities were noted. His younger affected sibling was found to have similar biochemical findings at two years old.

Five patients have been reported in the *cblF* group. Two females were small for gestational age with MMA, poor feeding, growth retardation and persistent stomatitis (63,193). One had minor facial anomalies, dextrocardia, and abnormal Cbl absorption from the gut. The other had persistent rash, macrocytosis and elevated homocysteine and died suddenly despite a good response to Cbl therapy. Another case had recurrent stomatitis in infancy, arthritis at age four, confusion, disorientation and pigmentary dermatitis at age ten (194). Another had aspiration pneumonia at birth, hypotonia, lethargy, hypoglycemia, thromobocytopenia, and neutropenia. A Canadian girl was diagnosed at six months because of anemia, failure to thrive, developmental delay, recurrent infections, low serum Cbl, and Cbl malabsorption (195).

1.9.7 Laboratory Findings in Combined Methylmalonic Aciduria & Homocystinuria

The methylmalonic aciduria in children with combined MMA and homocystinuria is distinctly less severe than that encountered in children with isolated mutase deficiency. Elevations of homocysteine may be unremarkable in some *cblC* patients, even when acutely ill. One *cblF* patient had no detectable homocystinuria despite a cellular deficit in methionine synthase activity, although others with *cblF* have shown homocystinuria (63,193,195,194). Homocysteine levels in *cblC* patients have not been well documented although it may be comparable to homocysteine levels seen in patients with MTHFR deficiency, a disease that also leads to homocystinuria due to an inability to convert 5,10-methyleneTHF to 5-methylTHF, a critical methyl donor for synthesis of MeCbl in the reaction that converts homocysteine to methionine. Homocysteine levels in MTHFR deficiency range from 15 to 667 μM/24 h with a mean of 130 μM/24 h. Homocysteine is not normally detected in urine or free in plasma and was found in patients plasma with a mean value of 57 μM (range: 12 to 233 μM). Total plasma or serum homocysteine data are scarce; levels of 60 to 184 μM (controls: 4 to 14 μM) have been reported (196,197,198,186).

1.10 Treatment & Prognosis

Pharmacologic doses of Cbl can in many cases reverse methylmalonic aciduria. Individuals with *cblA* or *cblB* disease generally respond to Cbl therapy whereas individuals with mut deficiency generally do not. A diet restricted in protein plus supplementary Cbl and oral antibiotic therapy has improved alertness and appetite, decreased vomiting, and resulted in growth acceleration and improved behavior (199,200). Two treatment regimens for children with MMA exist and are used in tandem. A restricted protein diet or a special formula restricted in amino acid precursors of methylmalonate should be instituted as well as supplementary Cbl (1-2 mg CN-Cbl or, preferably, OH-Cbl intramuscularly daily for several days.) L-carnitine supplements may be a useful therapeutic adjunct in patients with methylmalonic acidemia, presumably by repleting intracellular and extracellular stores of free carnitine that are depleted in affected patients because of exchange with excess methylmalonyl CoA and propionyl CoA.

Exogenous Cbl deficiency will respond dramatically to physiologic amounts of Cbl and certain forms of homocystinuria will also respond to supplements of pyridoxine or folate. Successful treatement of *cblC*, *cblD* or *cblF* patients may demand administration of very large amounts (up to 1mg daily) of OHCbl (49,201,202,203,204). This treatment has resulted in dramatic decreases in urinary methylmalonate and less dramatic decreases in urinary homocystine (205). Supplementation with betaine can reduce homocysteine levels and restore methionine (206). Early diagnosis and prompt institution of therapy with Cbl supplements (and betaine) may be the only way to change the outcome of these patients. Delayed treatment can result in incompletely reversible developmental delays or neurologic deficits. In the case of *cblC* patients, therapy has been unsuccessful for the most part. As a group, the *cblF* patients have responded well to treatment with Cbl. Treatment regimen includes intramuscular injection of 1mg OHCbl per day followed by a reduction to 1mg 1-3 times a week. Macrocytic anemia has responded to folinic acid therapy (129). General improvements are seen after 1-3 weeks but neurologic symptoms and developmental problems are slower to improve at about 3-4 months of therapy. One *cblE* patient was diagnosed prenatally, treated with OHCbl in utero and postnatally and developed normally with only minor clinical symptoms (188,207). Betaine therapy, previously used as an adjunct to Cbl therapy, is now the therapy of choice in addition to injections of OHCbl. Other adjuncts may be tried including supplementation with betaine, methionine, carnitine and pyridoxine (188).

1.11 Thesis Objectives

This thesis has two major components: 1) The study of mutations in *MMAA* and *MMAB* genes and 2) the identification of the *MMACHC* gene.

1) The recent discovery of two genes, *MMAA* and *MMAB*, responsible for the *cblA* and *cblB* forms of isolated methylmalonic aciduria, respectively, has allowed for the molecular dissection of these diseases. The largest collection of these patient cell lines in the world is available and maintained at the Repository for Mutant Human Cell Strains, Montreal Children's Hospital). This resource has allowed for the extraction of DNA from these individual cell lines and a detailed analysis of the spectrum of mutations in patients with these diseases. The fundamental importance of this work is the understanding of

disease mechanism, mutation history, the behaviour of mutants in cell culture, correlations of genotype with phenotype, how mutations manifest as disease in affected individuals, and ultimately to the understanding of the role of cobalamin in cellular processes and possibly to improved treatment of patients with these diseases. Chapter 2 describes the spectrum of mutations in the *MMAA* gene identified in *cblA* patient cell lines. Chapter 3 deals with the identification of mutations in the *MMAB* gene and their relationship to *cblB* disease.

2) The objective to identify the gene responsible for *cblC* disease stemmed from the collection of a large number of patient cell lines with *cblC* disease. Over 215 *cblC* patient cell lines were available, including cells from members of four families. In 2002, the results of a linkage scan performed on six families suggested linkage to chromosome 1p. This was the basis for further refining of the chromosomal interval by homozygosity mapping and sibling pair analysis, resulting in identification of the *MMACHC* gene. The gene responsible for *cblC* disease codes for a product that occupies an early step in cobalamin metabolism and mutations in this gene result in accumulation of both methylmalonic acid and homocystine, two byproducts of cobalamin-dependent metabolic processes. Much has been learned from simply studying the clinical phenotype of the 300 patients diagnosed with *cblC* in the 37 years since the orignal description of *cblC* disease (48). This work led to the identification of the spectrum of mutations in *cblC* patients, analysis of phenotype-genotype correlations, identification of functional domains and the beginning of the dissection of specific vitamin B_{12} related cellular processes. The immediate impact has been the translation of these findings into molecular diagnostic tests in families where mutations are known. Chapter 4 and 5 report the identification of the *MMACHC* gene, the identification of novel mutations, population genetic and functional analyses, and genotype-phenotype findings in *cblC* individuals.

CHAPTER 2.0: Molecular basis of methylmalonic aciduria type A

2.0 Identification of the gene responsible for *cblA*: *MMAA*

The identification of the gene responsible for the *cblA* type of cobalamin disease has led to great advances in our understanding of this disease and in the processes of cobalamin metabolism. The knowledge of cobalamin utilization in bacterial systems and the availability of the Clusters of Orthologous Groups (COG) database allowed for the identification of this gene, as described above, which was called *MMAA* for methylmalonic aciduria, *cblA* type (152). A search for MCM in the COG database yielded COG2185, which was a member of a cluster of genes in 16 complete bacterial genomes. In 10 of these, COG1703 was found immediately adjacent to COG2185 and was characterized as a putative periplasmic protein kinase, ArgK, related to the G3E family of GTPases. One member of this COG, ArgK, was described as a coupled ATPase of the lysine-arginine-ornithine (LAO) transport system in E. coli (164). This candidate (COG1703) was pursued as a candidate gene involved in AdoCbl metabolism, given its frequency and proximity to MCM (COG2185) within operons. A search for human expressed sequence tags was carried out using the sequence of COG1703 from *Archeoglobus fulgidus* (from COG1703 GenBank accession no.AAB89957) and yielded 28 ESTs in Unigene clusters Hs.126216 and Hs.21017. A composite cDNA was constructed by alignment of ESTs. Orthologues were identified in mouse, *C. elegans*, *M. tuberculosis* and *E. coli*. A multiple tissue Northern blot using a 508 bp probe demonstrated the presence of three RNA species of ~1.4, 2.6 and 5.5 kb with highest levels of expression in liver and skeletal muscle. The 1.4 kb transcript is similar to the predicted length of the composite cDNA and this transcript predominated in liver whereas the larger 5.5 kb transcript predominated in skeletal muscle. Five deleterious mutations in four *cblA* patients confirmed the authenticity of the gene assignment.

Following the identification of the *MMAA* gene, to further characterize the genetic basis of disease in *cblA* patient cell lines, genomic DNA was extracted from 37 *cblA* cell lines, the total number available at the time of this study. These patients had been diagnosed by complementation analyses and biochemical studies in cell culture. Twenty-two mutations were identified, genotype-phenotype correlations were examined,

molecular diagnostic tests were designed for each mutation. Haplotype analysis suggested a common ancestor for the most frequent mutation identified in patient samples.

2.1 Mutations in the MMAA gene in patients with the cblA disorder of vitamin B_{12} metabolism

Jordan P Lerner-Ellis[1], C Melissa Dobson[6], Timothy Wai[1], David Watkins[1], Jamie C Tirone[1], Daniel Leclerc[1], Carole Doré[5], Pierre Lepage[5], Roy A Gravel[6], David S Rosenblatt[1,2,3,4].

Department of [1]Human Genetics, [2]Biology, and [3]Medicine, McGill University, [4]McGill University Health Centre, [5]McGill University and Genome Quebec Innovation Centre, Montreal, QC, Canada. [6]Department of Biochemistry and Molecular Biology, University of Calgary, AB, Canada.

Published: Human Mutation 2004 Dec;24(6):509-16.

ABSTRACT

Mutations in the *MMAA* gene on human chromosome 4q31.1-2 result in vitamin B_{12}-responsive methylmalonic aciduria (*cblA* complementation group) due to deficiency in the synthesis of adenosylcobalamin. Genomic DNA from thirty-seven *cblA* patients, diagnosed on the basis of cellular adenosylcobalamin synthesis, methylmalonyl CoA mutase function, and complementation analysis, was analyzed for deleterious mutations in the *MMAA* gene by DNA sequencing of exons and flanking sequences. Eighteen novel mutations were identified bringing the total number of mutations identified in thirty-seven *cblA* patients to twenty-two. Thirteen mutations result in premature stop codons; three are splice site defects; and six are missense mutations that occur at highly conserved residues. Eight of these mutations were common to two or more individuals. One mutation, c.433C>T (R145X), represents 43% of pathogenic alleles. Restriction endonuclease or heteroduplex diagnostic tests were designed to confirm mutations. None of the sequence changes identified in *cblA* patients were found in one hundred alleles from unrelated control individuals.

Databases

***MMAA* – Genbank: NM_172250.1, NT_016606.16, MIM#607481, MIM#251100**

Key Words

Vitamin B_{12}, Cobalamin, Methylmalonic aciduria, *cblA*, *MMAA*.

INTRODUCTION

Methylmalonic acidemia is a rare human disorder caused by a decreased activity of mitochondrial methylmalonyl CoA mutase (MCM), one of two mammalian enzymes that utilize vitamin B_{12} (cobalamin, Cbl) derivatives. MCM catalyzes the adenosylcobalamin (AdoCbl)-dependent rearrangement of L-methylmalonyl-CoA to succinyl-CoA. This is an important intermediary step in the catabolism of branched chain amino acid and odd chain fatty acids via the tricarboxylic acid cycle. A defect in either MCM or the gene products involved in the conversion of exogenous Cbl to AdoCbl leads to the accumulation of methylmalonic acid in the blood and urine of affected individuals.

The *cblA* complementation class of inborn errors of cobalamin metabolism (MIM#251100) is one of three known disorders that affect AdoCbl synthesis without also affecting the synthesis of methylcobalamin (MeCbl), a second Cbl derivative required for activity of the cytoplasmic enzyme methionine synthase (Rosenblatt & Fenton., 2001). The gene responsible for *cblA* has been identified through the examination of prokaryotic gene arrangements and is called *MMAA* (MIM#607481) (Dobson et al., 2002). The identification of four mutations in five *cblA* patients confirmed the gene assignment. The precise role of the *MMAA* gene product is not known, but previous studies have suggested its involvement in either mitochondrial Cbl transport or reduction of cobalamin (Fenton & Rosenberg., 1978; Dobson et al., 2002). More recently a role for *MMAA* in the maintenance of MCM dimer stabilization and cofactor protection has been suggested (Korotkova & Lidstrom. 2004).

Individuals affected with *cblA* typically present with severe disease in infancy or early childhood and are prone to potentially life threatening acidotic crises (Rosenblatt & Fenton., 2001). Cultured fibroblasts from *cblA* patients have characteristic biochemical features which include: 1) reduced synthesis of AdoCbl from exogenous cyanocobalamin (CNCbl) with normal levels of methylcobalamin (MeCbl); 2) reduced ability to incorporate propionate into cellular macromolecules, an indirect measure of AdoCbl-dependent MCM activity; and 3) the ability to complement all other inborn errors of

cobalamin metabolism in cell culture (Gravel et al., 1975; Fenton & Rosenberg., 1978; Willard et al., 1978; Watkins et al., 2000).

Thirty-seven *cblA* patients were identified on the basis of AdoCbl synthesis, MCM function and complementation analysis. Our aim was to identify novel mutations in the *MMAA* gene in these patients by sequencing the exons and flanking regions.

MATERIALS & METHODS

Patients

Sequencing of the *MMAA* gene (NM_172250.1) was carried out on a panel of thirty-seven *cblA* patients and fifty anonymous controls. This includes five patients previously studied by Dobson et al. (2002). Patient cell line numbers are listed in Table 1 along with the sex, race, and age of onset of these individuals except for three patients for whom complete clinical information was not available. Consanguinity was reported in two individuals.

Fibroblast Studies

Diagnosis of *cblA* was based on assessment of cellular AdoCbl synthesis and MCM function, and was confirmed by complementation analysis (Watkins et al., 2000). MCM function in intact fibroblasts was assessed by measuring incorporation of [^{14}C]propionate into acid-precipitable material. Cell lines were plated into 35-mm tissue culture dishes at a density of 4 x 10^5 cells per dish and incubated for 18 hours in Puck's F medium supplemented with 15% FBS and 100 µmol/L [^{14}C]propionate (New England Nuclear) diluted with unlabelled propionate to give a final specific activity of 10 µCi/µmol. The assay was performed in the presence and absence of 3.75 µmol/L OHCbl. Macromolecules were precipitated with 5% trichloroacetic acid. The precipitated material was dissolved in 0.2N sodium hydroxide and radioactivity was determined by liquid scintillation counting. All patient fibroblasts had decreased incorporation of label from [^{14}C]propionate into cellular macromolecules compared to control fibroblasts (Table 2).

Cobalamin distributions were determined by growing cells in 25 pg/ml [^{57}Co]CNCbl (Amersham or ICM) for four days followed by extraction of labeled cobalamins in darkness in absolute ethanol at 85°C. Intracellular cobalamins were separated by high performance liquid chromatography as previously described (Rosenblatt et al., 1984). All patient fibroblast lines had decreased synthesis of AdoCbl from [^{57}Co]CNCbl compared to controls (Table 2).

DNA Sequencing

DNA was extracted from patient and control fibroblasts using the Qiagen genomic DNA extraction kit for cultured cells. *MMAA* exons 1 - 3 and flanking sequences were amplified by PCR using primers listed in Table 3 (primers were designed using Primer 3.0 software available online at http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and exons 4 - 7 were amplified using primers described by Dobson et al. (2002). PCR products were purified with Montage PCR_{96} filter plates from Millipore. Purified PCR products were used for 10 µl sequencing reactions made up of 2 µl PCR product, 1 µl of BigDye Terminator Cycle Sequencing Version 2.0 or Version 3.0 (Applied Biosystems), 1.5 µl of 5X buffer, 5 µl H_2O, and 0.5 µl [1µM final] of sense or anti-sense primer. Amplicons were sequenced in both forward and reverse directions. Products were analyzed on an ABI3700 automated DNA sequencer (Applied Biosystems). Gel files were processed using Sequence Analysis software (PE Applied Biosystems) and were assembled and analyzed using the Phred/Phrap/Consed System (Ewing et al., 1998; Gordon et al., 1998). DNA mutation numbering is based on cDNA sequence with +1 corresponding to the A of the ATG translation initiation codon.

Restriction Endonuclease and Heteroduplex analysis

MMAA sequence changes identified by sequence analysis were confirmed by restriction endonuclease or heteroduplex analysis. Restriction endonucleases were purchased from New England Biolabs. Ten sequence changes resulted in either the creation or loss of a naturally occurring restriction site. Five sequence changes were

confirmed by analysis using artificially created restriction sites. Digestions were performed under conditions recommended by the enzyme manufacturer. Five sequence changes (one duplication and four deletions) detected by sequencing were confirmed by heteroduplex analysis of PCR products. PCR products were denatured for 4 minutes at 94°C prior to electrophoresis on 8% polyacrylamide gels (29:1 acrylamide:bisacrylamide). Table 3 describes the primers and restriction enzymes used to detect sequence changes.

RESULTS

Two mutations in the *MMAA* gene coding sequence and/or flanking region were identified in thirty-five *cblA* patients and a single mutation was identified in two patients. None of these mutations was detected in one hundred control alleles. Mutations were identified in exons 2-7. No mutations were detected in exon 1, which is likely untranslated due to the presence of an in-frame stop codon that precedes the first in-frame ATG in exon 2.

Novel MMAA Mutations

Mutations are listed in Table 1 and Figure 1. Details of mutation confirmation analysis are described in Table 3.

c.64C>T (R22X) – This C>T sequence change at position c.64 results in a nonsense mutation by changing an arginine codon at position 22 to a stop codon in exon 2 and is common to both WG1449 and WG1588 cell lines in heteroallelic form.

c.161G>A (W54X) – Cell line WG2578 is heteroallelic for a G>A sequence change at c.161 which results in the substitution of a stop signal for tryptophan at codon 54 in exon 2.

c.266T>C (L89P) – A T>C nucleotide change at position c.266 in exon 2 results in the substitution of proline for leucine at codon 89. The leucine residue is conserved between mouse and human and occurs within the mitochondrial leader sequence, which is not present in orthologous polypeptides of *Escherichia coli*, *Mycobacterium tuberculosis* and *Archeoglobus fulgidus*. This change was detected in two cell lines, WG1449 and WG2578, in heteroallelic form.

c.387C>A (Y129X) – Cell line WG3009 is homoallelic for a nonsense mutation in exon 2 at position c.387 due to a C>A nucleotide change. This results in the replacement of tyrosine by a stop codon at amino acid 129.

c.433C>T (R145X) – This C>A sequence change occurs in exon 2 at position c.433 and it accounts for 31 of 72 (~43%) pathogenic alleles in this panel of *cblA* patients. Arginine is converted to a stop signal at codon 145. Ten patients are homoallelic and eleven patients are heteroallelic for this mutation (Table 1). This mutation was not detected in 182 control alleles examined.

c.434G>A (R145Q) – WG1191 is homoallelic for a G>A nucleotide change at position c.434. Arginine is converted to a glutamine at codon 145. This residue is conserved between human, mouse, *E. coli* and *M. tuberculosis* but not with *A. fulgidus* which has valine at this position, five amino acids upstream of the Walker A motif.

c.439+1_4delGTCA – This deletion results in a splice site defect. Four bases, GTCA, are deleted at the exon 2 donor splice site. WG1776 is heteroallelic for this sequence change.

c.440G>A (E147G) – The first nucleotide of exon 3, c.440, is converted from G>A resulting in the substitution of glycine for glutamic acid at amino acid position 147. This amino acid is conserved between human, mouse, *E. coli*, *M. tuberculosis* and *A. fulgidus* and it is three amino acids upstream of the Walker A motif. Cell line WG1518 is heteroallelic for this sequence change.

c.450_451insG – An insertion of a guanine nucleotide at cDNA position 450-451 of exon 3 results in a frame shift mutation and creation of a stop signal downstream at amino acid position 169. Cell lines WG1518, WG2185 and WG2893 are heteroallelic for this insertion.

c.503delC – A cytosine deletion was detected at position c.503 in exon 3. A truncated protein product is predicted from this mutation. Cell line WG3003 is heteroallelic for this mutation.

c.653G>A (G218E) - Cell lines WG1588 and WG2188 are both heteroallelic for a point mutation at position c.653 in exon 4, a G>A change that results in a glycine to a glutamic acid substitution at codon 218. The glycine residue is highly conserved between human, mouse, *E. coli*, *M. tuberculosis* and *A. fulgidus*. This residue is located between the predicted Mg^{2+} binding site and Walker B motif of the polypeptide chain.

c.733+1G>A – Cell line WG2623 is homoallelic for a G>A nucleotide change at splice site position c.733+1 in intron 4. Disruption of *MMAA* transcript processing is predicted.

c.742C>T (Q248X) – Sequence analysis of DNA from cell line WG2230 revealed a heteroallelic point mutation at position c.742 in exon 5. A C>T nucleotide change results in the substitution of a stop signal for a glutamine residue at codon 248.

c.959G>A (W320X) – A homoallelic nonsense mutation was found in cell line WG2627 at codon 320 of exon 6 as the result of a G>A sequence change at position c.959.

c.970-2A>T – WG1798 is heteroallelic for a splice site mutation at position -2 of the intron 6 / exon 7 boundary.

c.988C>T (R330X) – The WG2063 cell line is homoallelic for a C>T nucleotide change at position c.988 in exon 7, resulting in the creation of a nonsense mutation at codon 330. The mutation is 37 amino acids downstream of a predicted GTP binding site.

c.1076G>A (R359Q) – Sequence analysis of DNA from WG3084 revealed a heteroallelic G>A change at nucleotide position c.1076 in exon 7. Cell line WG2704 is homoallelic for this sequence change. This alteration results in the conversion of the normal arginine codon to a glutamine codon at position 359, resulting in a charge change at this site. The c.1076 guanine nucleotide is highly conserved between human, mouse, *E. coli*, *M. tuberculosis* and *A. fulgidus*.

c.1089_1090delGA – This mutation occurs in exon 7. Guanine and adenine are deleted at positions c.1089_1090 resulting in a frameshift. Cell line WG2188 is heteroallelic for this mutation.

MMAA mutations [c.260_267dupATAAACTT, c.283C>T (Q95X), c.592_595delACTG and c.620A>G (Y207C)] previously identified by Dobson et al. (2002) were confirmed in the originally described patients and tested for in the remainder of the thirty-two patient cell lines. The nomenclature of mutations, c.260_267dupATAAACTT and c.592_595delACTG, have been changed from their original designation (Dobson et al., 2002) to adhere to current guidelines (den Dunnen & Antonarakis. 2000). The c.283C>T (Q95X) mutation, initially reported in WG1776, was also observed in one additional cell line: WG1798. The c.592_595delACTG frameshift mutation was originally detected in cell lines WG1192, WG2014 and WG3080. This sequence change was also observed in three additional cell lines: WG2664, WG1516 and WG2922. WG1192 is homoallelic and the five other cell lines are heteroallelic.

SNPs

Five single nucleotide polymorphisms (SNPs) were observed in intronic regions: c.1-56A>G (rs4835011), c.439+108A>G (rs4835012), c.734-74G>A (rs11721510), c.820-169T>C (rs2279717) and c.820-110A>G. SNPs c.734-74G>A and c.820-110A>G

were not previously documented. Table 4 lists the allelic frequencies of SNPs in patients and controls. Interestingly, c.820-110A>G was not found in any of the controls from our panel but is present in thirty-one of seventy-four patient alleles, all in patients with the c.433C>T mutation. A sequence change at position c.1089G>C (rs2270655), that results in a Q363H amino acid change, was identified in four of one hundred control alleles in heteroallelic form. A silent sequence change, c.747G>A (S249S), was detected in homoallelic form in cell line WG2627, and in six of one hundred control alleles in heteroallelic form.

Haplotype Analysis

Haplotypes were constructed from genotype data for patients homoallelic for the c.433C>T and c.592_595delACTG mutations using five SNP markers detected by sequencing analysis (c.1-56A>G (rs4835011), c.439+108A>G (rs4835012), c.734-74G>A (rs11721510), c.820-169T>C (rs2279717) and c.820-110A>G. These markers span 14,801 bp from intron 1 to intron 5. A common haplotype was observed in patients with each of these mutations (Fig. 2).

DISCUSSION

In this study we report the identification of eighteen novel mutations in the *MMAA* gene of *cblA* patients. Four previously identified disease-causing mutations in *MMAA* reported in five *cblA* patients (Dobson et al., 2002) were confirmed and second mutations were identified in two of these patients (WG1776 and WG2014). In total, twenty-two mutations have been identified: thirteen result in premature stop codons, including three deletions, one insertion and one duplication; three are splice site defects; and six are missense mutations that occur at highly conserved residues. Two mutations were identified in thirty-five patients, consistent with autosomal recessive inheritance of the *cblA* disorder, and one mutation was identified in two patients. None of the sequence changes identified in *cblA* patients was found in a minimum of one hundred alleles from unrelated control individuals.

Common Mutations

Several mutations were identified in more than one individual. The c.433C>T (R145X) mutation was identified in twenty-one patients and this sequence change accounts for ~42% of pathogenic alleles in this panel of *cblA* patients. All patients homozygous for the c.433C>T mutation were also homozygous at five SNPs within the *MMAA* gene (Fig. 2), allowing definition of a common haplotype associated with this mutation. The c.820-110A>G SNP was seen only in patients carrying the c.433C>T mutation and not detected in any controls from our sample. Genotypes of patients heterozygous for the c.433C>T mutation were also consistent with occurrence of c.433C>T on the same haplotype. This mutation was observed primarily in patients of European ancestry, but was also detected in heterozygous form in a Black individual and one of mixed Asian and European origin. These data suggest a common ancestor for the c.433C>T mutation.

The c.592_595delACTG mutation was identified in six individuals, five of whom were of European origin; information on the ethnic background of the sixth patient was unavailable. WG1192 is homozygous for this deletion and was also homozygous across five intronic SNPs (Fig. 2), allowing inference of a haplotype associated with this mutation. The genotypes of the remaining five patients who carry this deletion in heterozygote form are consistent with the mutation being on the same haplotype as WG1192. Four patients were compound heterozygote for c.592_595delACTG and c.433C>T mutations. Genotypes of these individuals were consistent with the haplotypes defined in patients homozygous for these mutations.

Other common mutations identified in more than one individual include c.64C>T (R22X), c.266T>C (L89P), c.653G>A (G218E), c.283C>T (Q95X), c.450_451insG, and c.1076G>A (R359Q). Further study will be necessary to determine if common mutations are being transmitted on the same genetic background.

Genotype-Phenotype Correlation

No direct correlations could be made between the mutations identified in the *MMAA* gene and clinical severity or the degree of impairment of cobalamin metabolism observed in tissue culture studies. Clinically, there was not much variation in presentation among the *cblA* patients in the present study. All patients presented during infancy or childhood with signs of metabolic acidosis. Variation in age of onset may be at least partly explained by environment, since in several cases patients with childhood onset were reported to be self-limiting their protein intake prior to diagnosis. The age of onset of patients homozygous for the c.433C>T mutation varied from the neonatal period to ten years. This supports the important role of environment, genetic modifiers, or both, in the clinical presentation of the *cblA* disorder. Interestingly, of all the vitamin B_{12} inborn errors of metabolism, the *cblA* patients are the most responsive clinically to vitamin B_{12} supplementation.

The *MMAA* gene has four sequence motifs which define the ArgK subfamily of G3E GTPases, including the Walker A motif, a Mg^{2+}-binding aspartate residue, the Walker B motif, and a GTP-binding motif, as described by Leipe et al. (2002). The function of the *MMAA* gene product is not known and it is currently not possible to predict functional effects of missense mutations. A role for the *MMAA* gene product has recently been suggested. A *MMAA* homologue, meaB from *Methylobacterium extorquens*, was hypothesized to protect MCM from irreversible inactivation. MeaB was shown to bind MCM and suggested to stabilize the dimer form of the enzyme and/or to protect bound cofactor from attack by oxygen, water, and highly reactive radical intermediates (Korotkova & Lidstrom. 2004). Further investigation of defects in *MMAA* will be critical to our understanding of the human *MMAA* gene product and to its role in patients with the *cblA* disorder.

ACKNOWLEDGEMENTS

We offer many thanks to the clinicians who provided patient samples and clinical information, to Nora Matiaszuk for complementation analysis, to Gail Dunbar for growth

of patient fibroblasts, to Brian Gilfix, Maria Galvez, Antonia Klitorinos, Angela Hosack, Lisa Worgan, Kirsten Niles and Emily Moras for assistance, and to Vince Forgetta for quality control of sequence data. This work was supported by an operating grant from the Canadian Institutes for Health Research (CIHR) and by a CIHR Group Grant in Medical Genetics. This is a publication of the Hess B. and Diane Finestone Laboratory in memory of Jacob and Jenny Finestone.

REFERENCES

den Dunnen, J.T., Antonarakis, S.E. 2000. Mutation nomenclature extensions and suggestions to describe complex mutations: a discussion. Hum Mutat 15: 7-12.

Dobson, C.M., Wai, T., Leclerc, D., Wilson, A., Wu, X., Doré, C., Hudson, T., Rosenblatt, D.S., Gravel, R.A. 2002. Identification of the gene responsible for the *cblA* complementation group of vitamin B_{12}-responsive methylmalonic acidemia based on analysis of prokaryotic gene arrangements. Proc Natl Acad Sci USA 99: 15554-15559.

Ewing, B., Hillier, L., Wendl, M., Green, P. 1998. Basecalling of automated sequencer traces using phred I. Accuracy assessment. Genome Res 8: 175-185.

Fenton, W.A., Rosenberg, L.E. 1978. Genetic and biochemical analysis of human cobalamin mutants in cell culture. Annu Rev Genet 12: 223-248.

Gordon, D., Abajian, C., Green, P. 1998. Consed: A graphical tool for sequence finishing. Genome Res 8: 175-185.

Gravel, R.A., Mahoney, M.J., Ruddle, F.H., Rosenberg, L.E. 1975. Genetic complementation in heterokaryons of human fibroblasts defective in cobalamin metabolism. Proc Natl Acad Sci USA 72: 3181-3185.

Korotkova, N., Lidstrom, M.E. 2004. MeaB is a component of the methylmalonyl-CoA mutase complex required for protection of the enzyme from inactivation. J Biol Chem. 279:13652-8.

Leipe, DD, Wolf, YI, Doonin, EV, Aravind, L. 2002. Classification and evolution of P-loop GTPases and related ATPases. J Mol Biol. 317:41-72.

Rosenblatt, D.S., Cooper, B.A., Pottier, A., Lue-Shing, H., Matiaszuk, N., Grauer, K. 1984. Altered vitamin B_{12} 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.

Rosenblatt, D., Fenton, W.A. 2001. Inherited disorders of folate and cobalamin transport and metabolism. In C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle, B. Childs, K.W. Kinzler, & B. Vogelstein (Eds.), The metabolic and molecular bases of inherited disease (8^{th} edition). (pp. 3897-3933). New York: McGraw-Hill.

Watkins, D., Matiaszuk, N., Rosenblatt, D.S. 2000. Complementation studies in the *cblA* class of inborn error of cobalamin metabolism: evidence for interallelic complementation and for a new complementation class (*cblH*). J Med Genet 37: 510-513.

Willard, H.F., Mellman, I.S., Rosenberg, L.E. 1978. Genetic complementation among inherited deficiencies of methylmalonyl-CoA mutase activity: Evidence for a new class of human cobalamin mutant. Am J Hum Genet 30: 1-13.

TABLES

Table 1. Summary of mutations found in *cblA* patients

Cell Line	Sex/Race	Age of Onset	Mutation 1	Predicted change	Mutation 2	Predicted change
WG1449	F/W	Neonatal	c.64C>T	R22X	c.266T>C	L89P
WG1588	F/B	6 weeks	c.64C>T	R22X	c.653G>A	G218E
WG2578	F/W	1 week	c.161G>A	W54X	c.266T>C	L89P
WG3080	F/W	1 week	c.260_267dup	Frameshift	c.592_595del	Frameshift
WG1776	F/W	7 months	c.283C>T	Q95X	c.439+1_4del	Splice site
WG1798	M/W	7 months	c.283C>T	Q95X	c.970-2 A>T	Splice site
WG3009	F/W	3 months	c.387C>A	Y129X	c.387C>A	Y129X
WG2019	M/Unknown	neonatal	c.433C>T	R145X	c.433C>T	R145X
WG1796	F/W	2 weeks	c.433C>T	R145X	c.433C>T	R145X
WG2653	F/W	2 weeks	c.433C>T	R145X	c.433C>T	R145X
WG1660	M/W	6 months	c.433C>T	R145X	c.433C>T	R145X
WG3038	M/W	7 months	c.433C>T	R145X	c.433C>T	R145X
WG1802	M/W	12 months	c.433C>T	R145X	c.433C>T	R145X
WG1761	F/W	14 months	c.433C>T	R145X	c.433C>T	R145X
WG2529	M/W	18 months	c.433C>T	R145X	c.433C>T	R145X
WG2037	M/W	4.5 years	c.433C>T	R145X	c.433C>T	R145X
WG3039	F/W	10 years	c.433C>T	R145X	c.433C>T	R145X
WG2893	Unknown	4 weeks	c.433C>T	R145X	c.450_451insG	Frameshift
WG2185	F/W	11 months	c.433C>T	R145X	c.450_451insG	Frameshift
WG3003	F/W/A	9 months	c.433C>T	R145X	c.503del	Frameshift
WG2664	M/W	4 days	c.433C>T	R145X	c.592_595del	Frameshift
WG2014	M/W	3 months	c.433C>T	R145X	c.592_595del	Frameshift
WG2922	M/W	5 months	c.433C>T	R145X	c.592_595del	Frameshift
WG1516	M/W	5 months	c.433C>T	R145X	c.592_595del	Frameshift
WG2230	F/B	12 months	c.433C>T	R145X	c.742C>T	Q248X
WG3084	M/W	9 months	c.433C>T	R145X	c.1076G>A	R359Q
WG1191	Unknown	Unknown	c.434G>A	R145Q	c.434G>A	R145Q
WG1518	M/W	6 months	c.440G>A	E147G	c.450_451insG	Frameshift
WG1192	Unknown	Unknown	c.592_595del	Frameshift	c.592_595del	Frameshift
WG1943	M/W	2 months	c.620A>G	Y207C	c.620A>G	Y207C
WG2188	F/Unknown	12 months	c.653G>A	G218E	c.1089_1090del	Frameshift
WG2623	M/W	4 days	c.733+1G>A	Splice site	c.733+1 G>A	Splice site
WG2627	M/W	3.75 years	c.959G>A	W320X	c.959G>A	W320X
WG2063	F/W	5 days	c.988C>T	R330X	c.988C>T	R330X
WG2704	M/W	6.5 years	c.1076G>A	R359Q	c.1076G>A	R359Q
WG2882	F/W	6 months	c.433C>T	R145X	ND	
WG1411	M/W	26 months	c.433C>T	R145X	ND	

Mutations in the *MMAA* gene (NT_016606.16) in patients with *cblA* are summarized. DNA mutation numbering is based on cDNA sequence: +1 corresponds to the A of the ATG translation initiation codon. In thirty-five patients two mutations were identified and in two patients one mutation was identified. Consanguinity was reported in cell lines WG2627 & WG2037. Cell lines are arranged sequentially by the position of the first mutation from 5' to 3' and then by the age of onset.

F = female; M = male; W = white; B = black; A = Asian

ND = No mutation detected

Table 2. ***cblA*** **patient fibroblast cobalamin distributions and propionate incorporation profiles**

Cell Line	AdoCbl (% of total intracellular cobalamin)	Prop. Inc. w/o OHCbl[1]	Prop. Inc. w/ OHCbl[2]
Control	15.3 ± 4.2 (n=3)	11 ± 4	11 ± 4
WG1449	3.4	2.3	13.0
WG1588	3.9	1.9	7.2
WG2578	6.1	0.7	3.3
WG3080	3.5	0.6	2.4
WG1776	6.6	2.3	4.6
WG1798	5.2	2.9	2.1
WG3009	1.5	1.3	2.2
WG2019	3.7	1.2	5.0
WG1796	3.7	1.5	4.4
WG2653	4.3	0.5	2.4
WG1660	2.8	2.5	6.6
WG3038	1.8	1.0	4.0
WG1802	3.5	0.4	3.3
WG1761	4.4	1.9	6.8
WG2529	1.5	0.5	3.0
WG2037	2.2	2.6	5.4
WG3039	2.8	3.0	5.9
WG2893	1.5	1.2	4.7
WG2185	5.0	1.0	2.0
WG3003	3.5	1.6	3.4
WG2664	3.1	1.2	6.7
WG2014	7.5	1.2	7.1
WG2922	1.4	0.9	2.3
WG1516	4.6	0.7	2.9
WG2230	4.0	1.9	4.7
WG3084	3.1	0.7	3.7
WG1191	5.0	0.7	N/D
WG1518	7.0	4.5	11.2
WG1192	5.0	1.3	N/D
WG1943	6.8	3.4	9.0
WG2188	3.4	0.9	2.6
WG2623	1.7	0.9	3.2
WG2627	1.9	0.5	3.5
WG2063	2.8	3.3	7.0
WG2704	6.1	0.9	3.0
WG2882	3.2	1.1	2.6
WG1411	2.3	1.6	6.1

[1] Prop. Inc. w/o OHCbl: Incorporation of [^{14}C]propionate (nmoles/mg protein/18hrs) without addition of OHCbl.

[2] Prop. Inc. w/ OHCbl: Incorporation of [^{14}C]propionate (nmoles/mg protein/18hrs) with the addition of 3.75μM OHCbl.

Control values (mean ± SD) is based on 12 determinations in 3 different controls.

N/D: Not done

Table 3. *MMAA* mutation confirmation

Mutation or Exon	Heteroduplex or Enzyme	Primers		PCR Product Size	Fragment Sizes[1]	
		5'-3' Sense	5'-3' Antisense	(bp)	Wild Type	Mutant
c.64C>T*	*NlaIV*	CCTAAAAGGCCTTTTAAGAGCACGGTTC[2]	2R[3]	412	412	386/26
c.161G>A†	*FokI*	2F[3]	2R[3]	515	298/<15	515
c.266T>C*	*BslI*	2F[3]	2R[3]	515	515	339/176
c.260_267dup	Heteroduplex	2F[3]	2R[3]	515	N/A	N/A
c.283C>T†	*StyI*	2F[3]	2R[3]	515	350/165	515
c.387C>A*	*MseI*	2F[3]	2R[3]	515	170/117/82/66/50/30	117/107/82/66/63/50/30
c.433C>T†	*TaqI*	GCCATGGAGGGAGTCTTCTC	TGGGATCCAGAGCAAGATTC	943	529/169/149/96	625/169/149
c.434C>T†	*TaqI*	GCCATGGAGGGAGTCTTCTC	TGGGATCCAGAGCAAGATTC	943	529/169/149/96	625/169/149
c.439+1_4del	Heteroduplex	GCCATGGAGGGAGTCTTCTC	TGGGATCCAGAGCAAGATTC	943	N/A	N/A
c.440G>A*	*Tsp509I*	GAACCCAGGGTGTTTCCTTC	CCTTCCTTTAGCGAGACCAA	457	194/104/93/68/52/45/30/11	194/93/78/68/52/45/30/26/
c.450_451insG	N/A[4]	3F[3]	3R[3]	586	N/A	N/A
c.503del	Heteroduplex	3F[3]	3R[3]	586	N/A	N/A
c.592-595del	Heteroduplex	4F[3]	4R[3]	507	N/A	N/A
c.620A>G*	*SphI*	4F[3]	4R[3]	507	507	384/123
c.653G>A†	*MnlI*	4F[3]	4R[3]	507	266/137/64/28/12	278/137/64/28/24
c.733+1G>A†	*HphI*	4F[3]	4R[3]	507	259/159/89	418/89
c.742C>T†	*PleI*	GTGATTTACAATTTCAGGTGTGAGT[2]	CCTTTGATTCAGGTATTTTAGCC	250	220/30	250
c.959G>A	N/A[4]	CGCAAACGTTCACAAGACTG[5]	ATGACTACACACTCCGTCTTTGTC[5]	185	163/22	185
c.970-2A>T†	*Eco0109I*	7F[3]	TCTCCACTTCGGGCAGAAATACGAAGGACC[2]	108	77/29	108
c.988C>T†	*ApaI*	TCGATAGGTAATTCGTATTTGGGCC[2]	GAATCTGTTCCCGGACTGTG	206	181/25	206
c.1076G>A†	*BsiEI*	7F[3]	CAAACTTTCTGTTGCTTCGGT[2]	210	186/24	210
c.1089_1090del	Heteroduplex	7F[3]	7R[3]	352	N/A	N/A
Exon 1	N/A	TCACCGGTCTGTCAAACGTA	TCCTGCCTGTCCACACATATC	707	N/A	N/A
Exon 2	N/A	GCCATGGAGGGAGTCTTCTC	TGGGATCCAGAGCAAGATTC	943	N/A	N/A
Exon 3	N/A	GAACCCAGGGTGTTTCCTTC	CCTTCCTTTAGCGAGACCAA	457	N/A	N/A

[1] Heteroallelic restriction cuts would have both fragment sizes observed.

[2] The underlined nucleotides denote changes made in the PCR primer to incorporate an artificial restriction site.

[3] Primer sequence is from Dobson et al. (2002).

[4] This sequence change was confirmed by sequence analysis only.

[5] Second primer used to confirm mutation by sequence analysis.

* The mutation results in the creation of a restriction enzyme site.

† The mutation results in the destruction of a restriction enzyme site.

N/A = Not applicable

Table 4. SNP allelic frequency

SNPs	Freq. in patient alleles	Freq. in normal alleles
c.1-56A>G (rs4835011)	5 / 56 (8.9%)	13 / 182 (7.1%)
c.439+108A>G (rs4835012)	3 / 56 (5.4%)	30 / 182 (16.5%)
c.734-74G>A (rs11721510)	11 / 74 (14.9%)	14 / 100 (14%)
c.747G>A (S249S)	2 / 74 (2.7%)	6 / 100 (6%)
c.820-169T>C (rs2279717)	12 / 74 (16.2%)	55 / 100 (55%)
c.820-110A>G	31 / 74 (41.9%)	0 / 100 (0%)
c.1089G>C (Q363H) (rs2270655)	0 / 74 (0%)	4 / 100 (4%)

Allelic frequency of SNPs within the *MMAA* gene in patients and controls as observed by sequence analysis in this study.

FIGURE LEGENDS

Figure 1. The exon structure of the *MMAA* gene (NT_016606.16) and the position of mutations identified with the corresponding amino acid change. The introns are drawn to scale except where "//" denotes a region longer than indicated. See Dobson et al (2002) for structural organization of the *MMAA* gene.

Figure 2. Haplotypes of patients with the c.433C>T (R145X) and c.592_595delACTG mutations. Haplotypes were constructed using the genotypes of patients for the polymorphisms: c.1-56A>G, c.439+108A>G, c.734-74G>A, c.820-169T>C, c.820-110A>G within the *MMAA* gene. Results are shown for patients that were homozygous for either c.433C>T (n = 10) or c.592_595del (n = 1) or heterozygous for both (n = 4). Patients carrying these mutations are listed in Table 1.

Figure 1

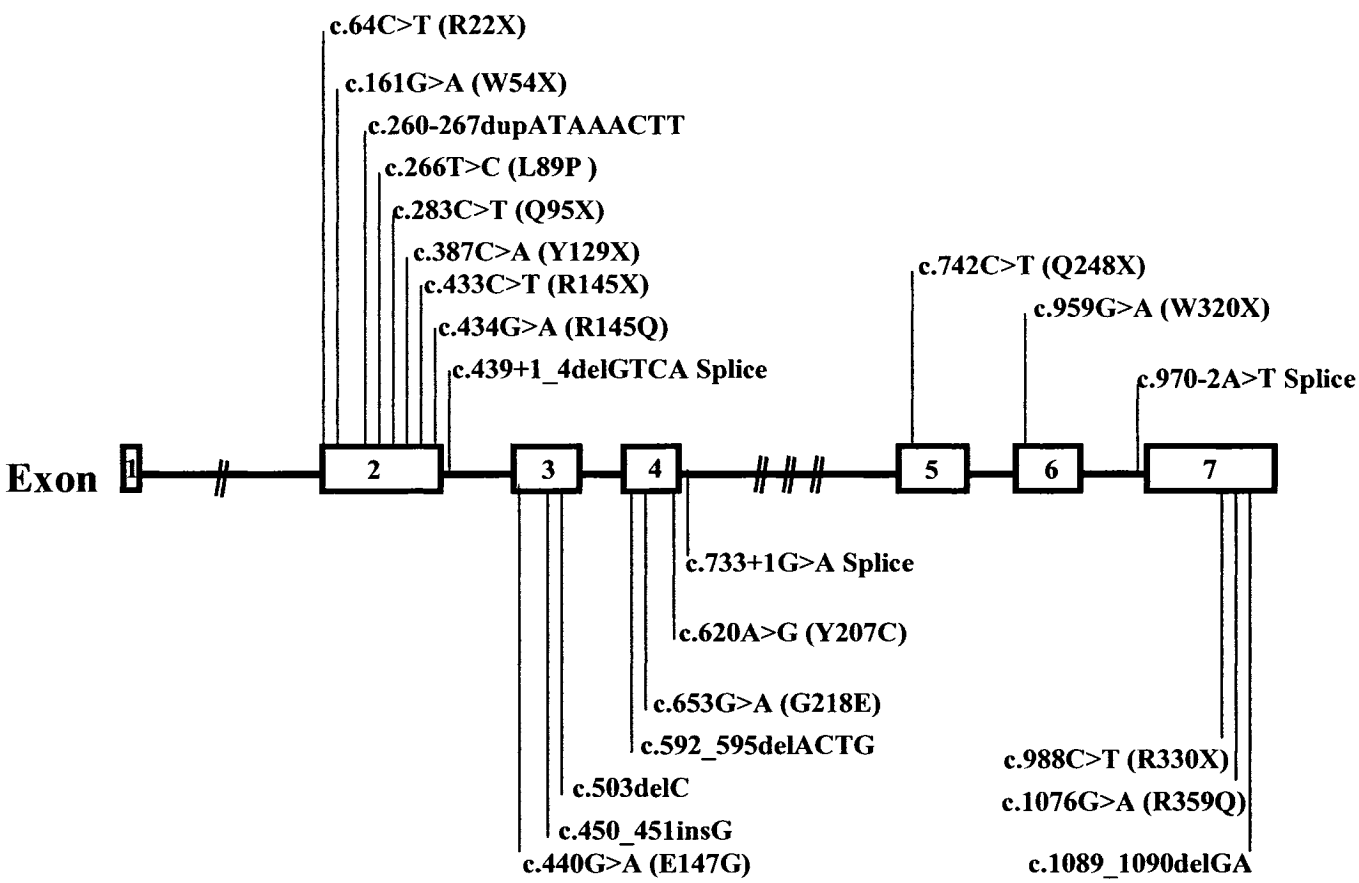

c.64C>T (R22X)
c.161G>A (W54X)
c.260-267dupATAAACTT
c.266T>C (L89P)
c.283C>T (Q95X)
c.387C>A (Y129X)
c.433C>T (R145X)
c.434G>A (R145Q)
c.439+1_4delGTCA Splice
c.742C>T (Q248X)
c.959G>A (W320X)
c.970-2A>T Splice
Exon
1
2
3
4
5
6
7
c.733+1G>A Splice
c.620A>G (Y207C)
c.653G>A (G218E)
c.592_595delACTG
c.503delC
c.450_451insG
c.440G>A (E147G)
c.988C>T (R330X)
c.1076G>A (R359Q)
c.1089_1090delGA

Figure 2

Sequence Change	c.433C>T		c.592_595del		c.433C>T / c.592_595del	
c.1-56A>G	A	A	A	A	A	A
c.439+108A>G	A	A	A	A	A	A
c.734-74G>A	G	G	G	G	G	G
c.820-169T>C	T	T	T	T	T	T
c.820-110A>G	G	G	A	A	G	A

CHAPTER 3.0: Molecular basis of methylmalonic aciduria, cblB type.

3.0 Identification of the gene responsible for the cblB complentation group of cobalamin metabolism

The gene responsible for the *cblB* complementation group was recently identified independently by two groups; Dobson *et al* (2002) (153) identified this gene by conducting homology searches using bacterial adenosyltransferases as query sequences and by using information derived from bacterial gene clusters through the Clusters of Orthologous groups (COG) database similar to the method used to identify the gene responsible for the *cblA* complementation group, MMAA (152). Leal *et al* (2003) identified and isolated clones from a bovine liver cDNA expression library that complemented an adenosyltranferase (ATR)-deficient bacterial strain (172).

Analysis of the COG database for clusters of genes containing methylmalonyl-CoA mutase yielded COG2185 which corresponds to the cbl-binding, carboxyl-terminal domain/subunit of MCM. Adjacent to COG2185 is COG1703, the orthologue of the *MMAA* gene. Adjacent to COG1703 is COG2096 (annotated as an uncharacterized ancient conserved region) in the *Archaeoglobus fulgidus* operon, which has sequence similarity to PduO, an ATP:cob(I)alamin adenosyltransferase from *Salmonella enterica.* This sequence was used as a template for BLASTP search of protein databases. This search yielded one hit corresponding to the N-terminal half of PduO from *Salmonella enterica* with 37% similarity (22%identity) (gi:5069458) and one hit to a full length protein in humans with 43% similarity (28% identity (gi:15080110). This protein was generated from a predicted mRNA supported by multiple (119) expressed sequence tags (Hs.12106). The mRNA varies at the 3' end with two predicted sizes of 1128 and 2361 nucleotides (BC005054 and BC011831 respectively), due to the use of alternate polyadenylation sites. Both transcripts have an open reading frame of 750bp, 250 codons with the same start and stop codons, differing only in the location of the polyadenylate track. More recently, several different alternatively spliced mRNAs products have been documented in the EST database. Seven different mutations, including two splice site mutation, one deletion and four missense mutations were identified in six *cblB* patient cell

lines by sequencing analysis of gDNA, confirming the authenticity of the gene assignment to *cblB* disease; the gene was called *MMAB* for methylmalonic aciduria, type *cblB*.

The calculated molecular mass of *MMAB* is 27.3 kDa. The gene consists of nine exons and encompasses ~18.9 kb of gDNA. Orthologous proteins were identified in mouse, *C. elegans, A. fulgidus and S. enterica*. These were aligned and compared and highly conserved regions were identified (153). This protein has a predicted mitochondrial leader sequence and cleavage signal site, consistent with early studies pointing to a mitochondrial defect in the human disorder. Analysis of a multiple human tissue Northern blot identified a single RNA species of 1.1 kb with highest levels of expression in liver and skeletal muscle. One mutation, c.556C>T (R186W), was observed in heteroallelic form in four of 240 control alleles, a frequency of 1/60. If this is the case, the prevalence of *cblB* disease would be expected to be much greater then is currently observed in the population. Replication of these findings in this group of controls and additional control cohorts is necessary as contamination may have been a contributing factor.

With the identification of the *MMAB* gene it was now possible to screen the cohort of *cblB* patient fibroblast lines available from the Repository for Mutant Human Cell Strains at the Montreal Children's Hospital (http://www.cellbank.mcgill.ca) for mutations in the *MMAB* gene. At the time of this study, 35 *cblB* patients had been diagnosed. Nineteen mutations were identified in total. Genotype phenotype correlations, haplotype analyses and mutation hotspots were among the findings and together have provided us with important information on the mechanism of disease, severity of mutations with respect to disease and active enzyme sites, and the history of these mutations. It is now possible to efficiently carry out molecular diagnostics on this gene.

3.1 Mutation and biochemical analysis of patients belonging to the cblB complementation class of vitamin B_{12}-dependent methylmalonic aciduria

Jordan P. Lerner-Ellis[1,6], Abigail B. Gradinger[1,6], David Watkins[1,6], *Jamie C. Tirone[1,6], Amélie Villeneuve[2], C. Melissa Dobson[3], Alexandre Montpetit[2], Pierre Lepage[2], Roy A. Gravel[3] and David S. Rosenblatt[1,4,5,6].

[1]Department of Human Genetics, McGill University, Montreal, Quebec, Canada

[2]McGill University and Genome Quebec Innovation Centre, Montreal, Quebec, Canada

[3]Department of Biochemistry and Molecular Biology, University of Calgary. Calgary, Alberta, Canada

[4]Departments of Biology and [5]Medicine, McGill University, Montreal, Quebec, Canada

[6]McGill University Health Centre, Montreal, Quebec, Canada

*deceased

Published: Mol Genet Metab. 2006 Mar;87(3):219-25.

ABSTRACT

Methylmalonic aciduria, cblB type (OMIM 251110) is an inborn error of vitamin B_{12} metabolism that occurs due to mutations in the MMAB gene. MMAB encodes the enzyme ATP:cobalamin adenosyltransferase, which catalyzes the synthesis of the coenzyme adenosylcobalamin required for activity of the mitochondrial enzyme methylmalonyl CoA mutase (MCM). MCM catalyzes the isomerization of methylmalonyl CoA to succinyl CoA. Deficient MCM activity results in methylmalonic aciduria and a susceptibility to life-threatening acidotic crises. The MMAB gene was sequenced from genomic DNA from a panel of 35 cblB patients, including five patients previously investigated. Nineteen MMAB mutations were identified, including 13 previously unknown mutations. These included 11 missense mutations, 2 duplications, 1 deletion, 4 splice site mutations and 1 nonsense mutation. None of these mutations was identified in one hundred control alleles. Most of the missense mutations (9 of 11) were clustered in exon 7; many of these affected amino acid residues that are part of the probable active site of the enzyme. One previously described mutation, c.556C>T (p.R186W), was particularly common, accounting for 33% of pathogenic alleles. It was seen almost exclusively in patients of European background, and was typically associated with presentation in the first year of life.

Key Words: cobalamin, vitamin B_{12}, cobalamin adenosyltransferase, cblB, MMAB, methylmalonic aciduria, inborn error of metabolism

INTRODUCTION

Methylmalonic aciduria, *cblB* type (OMIM 251110) is an inborn error of cobalamin (Cbl, vitamin B_{12}) metabolism characterized by accumulation of methylmalonic acid in blood and urine [1]. Patients typically present within the first year of life with lethargy, failure to thrive, recurrent vomiting, dehydration, respiratory distress and hypotonia, and are prone to life threatening acidotic crises; however, several patients have presented in childhood or adolescence [2]. Accumulation of methylmalonic acid results from decreased activity of the mitochondrial enzyme methylmalonylCoA mutase (MCM). This occurs either as the result of mutations affecting the *MUT* gene (*mut* complementation class), which encodes MCM, or mutations affecting genes encoding enzymes involved in synthesis of adenosylcobalamin (AdoCbl), which is required for activity of MCM (the *cblA* and *cblB* complementation classes). MCM catalyzes the isomerization of methylmalonylCoA to succinylCoA. This intermediary step is critical for normal catabolism of isoleucine, methionine, threonine, valine, odd chain fatty acids, and cholesterol via the tricarboxylic acid cycle, a process that occurs in the mitochondria.

The genes underlying the *cblA* and *cblB* disorders have been identified by analysis of prokaryotic gene arrangement. The function of the product of the *MMAA* gene, which is mutated in patients with the *cblA* disorder [3], remains unknown; roles in reduction of cob(II)alamin [4], as an accessory in mitochondrial membrane cobalamin transport [2], or in maintenance of MCM dimer stabilization [5] have been suggested. The *MMAB* gene, on chromosome 12q24.1, codes for the mitochondrial enzyme ATP:cobalamin adenosyltransferase (ATR), which catalyzes transfer of an adenosyl group from ATP to cob(I)alamin to form AdoCbl [6]. Deleterious mutations in *MMAB* were identified in six *cblB* patients. The gene was identified independently by Leal et al [7], who identified and isolated clones from a bovine liver cDNA expression library that complemented an ATR-deficient bacterial strain. These authors demonstrated decreased expression of ATR by Western blot analysis in fibroblasts from three *cblB* patients [7].

The human *MMAB* gene product is a member of the PduO family of cobalamin adenosyltransferases. Recently, the crystal structure of an *MMAB* homologue from the archaean *Thermoplasma acidophilum* (gi|16082403, TA1434) has been determined to a resolution of 1.5 Å [8]. The enzyme was shown to function as a homotrimer, with a

putative active site formed by interaction of adjacent subunits. Incorporation of two mutations described in human *cblB* patients into the *T. acidophilum* enzyme was shown to abolish adenosyltransferase activity *in vitro*. In addition to catalyzing adenosylation of cob(I)alamin, the *MMAB* gene product may also function as a chaperone, delivering AdoCbl in an activated state to MCM [9-11].

In the present study, we have sequenced the *MMAB* gene and surrounding intronic sequences in genomic DNA from a panel of 35 *cblB* patients.

MATERIALS & METHODS

Patients

Cultured fibroblasts from patients suspected of having an inborn error of cobalamin metabolism have been sent to our laboratory for clinical diagnosis and characterization for the past twenty-two years. Cell lines were assigned a unique identification code by The Repository for Mutant Human Cell Strains of the Montreal Children's Hospital (http://www.cellbank.mcgill.ca), and these codes were subsequently used. The referring physicians indicated whether cell lines should not be used to pursue research into the origin of these diseases. If physicians were contacted to request samples of DNA or cell lines from additional family members, informed consent was obtained from the patient or his/her family. This research protocol was approved by the Royal Victoria Hospital Research Ethics Board.

Studies were carried out on fibroblasts from 35 *cblB* patients (19 males; 14 females; 2 not specified). This panel included six cell lines (WG1185, WG2027, WG2186, WG2492, WG2523 and WG2633) previously sequenced by Dobson et al [6], as well as three lines (WG1680, WG1879 and WG2127) studied by Leal et al [7]. Results of cell culture studies of these fibroblast lines are shown in Table 1. The majority of these patients were reported to be of European ancestry with a subset of patients of Middle Eastern (six patients) and African (five patients) ancestry. Three patients were reported as offspring of consanguinous unions (1 first cousin, 1 second cousin, 1 not specified). Only unrelated patients were included in mutation analyses. Patient fibroblast lines had decreased incorporation of label from [^{14}C]propionate into cellular macromolecules (a

measure of MCM function in intact cells) and, in most cases, decreased synthesis of AdoCbl from exogenous [^{57}Co]CNCbl; in two patient fibroblast lines (WG1771 and WG2027), synthesis of AdoCbl fell within the control range. In all cases, the diagnosis of *cblB* was established by somatic cell complementation analysis [12,13].

DNA Sequencing

Sequencing of the MMAB gene (NM_052845) was carried out on thirty-five *cblB* patients and fifty unrelated CEPH controls. DNA was extracted using the Qiagen genomic DNA extraction kit for cultured cells. *MMAB* exons 1 - 9 and flanking sequences were amplified by PCR using primers described by Dobson et al [6]. PCR products were purified with Montage PCR96 filter plates from Millipore. Purified PCR products were used for 10 µl sequencing reactions made up of 2 µl PCR product, 1 µl of BigDye Terminator Cycle Sequencing Version 3.1 (Applied Biosystems), 1.5 µl of 5X buffer, 5 µl H_2O, and 0.5 µl [1µM final] of sense or anti-sense primer. Amplicons were sequenced in both forward and reverse directions. Products were analyzed on an ABI3730 Sequence Analyzer (Applied Biosystems). Sequencing files were processed using Sequence Analysis software (PE Applied Biosystems) and were assembled and analyzed using the Phred/Phrap/Consed System [14,15]. DNA mutation numbering is based on cDNA sequence with +1 corresponding to the A of the ATG translation initiation codon.

Haplotypes were determined in homozygous *cblB* patients using 10 SNPs within the *MMAB* gene that were sequenced during mutation analysis. The SNPs used were (from 5' to 3'): rs10774774 (exon 1), rs10774775 (exon 1), rs2287180 (exon 7), rs12309115 (exon 7), rs2287181 (exon 7), rs11610545 (exon 7), rs2287183 (exon 8), rs2287182 (exon 8), rs8228 (exon 9) and rs9593 (exon 9) (http://www.ncbi.nih.gov/SNP/). Frequency of haplotypes in control CEU samples (CEPH from Utah) were determined from the haplotype map developed by the International HapMap consortium using the program Haploview [16,17].

RESULTS

Results of mutation analysis in the panel of 35 *cblB* fibroblast lines are shown in Table 1. At least two mutations were identified in 34 patients; in the remaining patient

only a single mutation was identified. Three apparent mutations were identified in two patients (WG2487 and WG2492). Nineteen different *MMAB* mutations were observed in our panel. These include 11 missense mutations, 1 nonsense mutation, 1 duplication causing a frameshift, 1 duplication without a frameshift, 1 deletion causing a frameshift, and 4 mutations affecting splice sites. Of these mutations, 13 have not been previously described. In addition, several previously described mutations were observed in additional patients. Over half of the mutations (11 of 19) were localized to exon 7 (Figure 1).

Novel Mutations

The 13 novel mutations included 7 missense mutations as well as 1 nonsense mutation, 2 duplications, 1 deletion and 2 splice mutations. None of these sequence changes was identified in 100 control alleles. Two duplications (c.567_571dupCCGCC and c.563_577dupTGTGCCGCCGGGCCG) were identified in exon 7. Of these, c.567_571dupCCGCC (p.R191PfsX25) results in a frame shift and predicts a truncated protein product lacking part of the putative enzyme active site. The inframe insertion c.563_577dupTGTGCCGCCGGGCCG (p.186_190dup) would result in insertion of five additional amino acids within the active site. The c.585-2A>C mutation results in disruption of the splice acceptor site at the beginning of exon 8 and predicts an improperly spliced mRNA. The c.290G>A (p.G97E) mutation alters the final base of exon 3, part of the consensus splice site, and is predicted to affect proper splicing of the MMAB transcript. In addition, it would result in replacement of a glycine residue at position 97 of the protein by glutamic acid; this glycine is conserved in PduO cobalamin adenosyltransferases from eukaryotes, bacteria and archaea.

The c.700C>T (p.Q234X) mutation results in the creation of a stop codon at position 234, and is predicted to result in instability of the message or inactivity of the protein product.

The remaining novel mutations were missense mutations. Four of these (c.557G>A [p.R186Q], c.568C>T [p.R190C], c.569G>A [p.R190H] and c.572G>A [p.R191Q]) affected invariant arginine residues within the putative active site [8], and would be predicted to affect enzyme activity. Specifically, multiple amino acid substitutions at Arg191 have been shown experimentally to decrease or eliminate enzyme

activity in the T. acidophilum adenosyltransferase in vitro [8]. The c.521C>T (p.S174L) and c.539C>G (p.S180W) mutations result in replacement of residues in the region of the enzyme active site. While neither residue was identified as part of the active site [8], both were highly conserved in analysis of 22 adenosyltransferases from eukaryotes, bacteria and archaea [7].

Three different sequence changes (c.403G>A [p.A135T], c.571C>T [p.R191W] and c.656A>G [p.Y219C]) were observed in two patients in our panel. Two of these - c.403G>A (p.A135T) and c.571C>T (p.R191W) - were previously reported as mutations in WG2492 [6]. p.R191W abolishes adenosyltransferase activity in the T. acidophilum enzyme in vitro [8]. Of the other two mutations, p.Y219C affects a well conserved residue adjacent to three invariant residues identified as part of the active site (Glu214, Arg215 and Ser217), and presence of cysteine with its thiol group within the enzyme active site might be expected to deleteriously affect activity. The p.A135T change affects a residue that is poorly conserved [7,8].

Common mutations

The previously reported c.197-1G>T, c.291-1G>A, c.403G>A (p.A135T), c.556C>T (p.R186W) and c.571C>T (p.R191W) mutations [6] were observed in additional patients in our study. Overall, the c.556C>T (p.R186W) mutation was the most frequent in our panel of patients, accounting for 23 alleles (33% of all alleles in the panel). Other frequently observed alleles were c.700C>T (p.Q234X) (9 alleles), c.197-1G>T (7 alleles) and c.291-1G>A (4 alleles).

Among the common mutations, c.556C>T (p.R186W) was seen almost exclusively among patients of European descent (one homozygous patient was described as Mexican). All patients homozygous for the c.556C>T mutation shared a common haplotype constructed from 10 SNPs identified within the *MMAB* gene; however the same haplotype was also associated with other mutations (c.197-1G>T, c.291-1G>A, c.563_577dupTGTGCCGCCGGGCCG) in patients of various ethnic backgrounds. Two SNPs that were typed in these patients (rs2287182 and rs9593) were also typed by the HapMap Consortium (v.19). The haplotype associated with the c.556C>T mutation the

second most frequent haplotype observed in the CEU samples (CEPH, Utah), accounting for 13% of chromosomes in this population [16].

The c.197-1G>T splice site mutation was seen in heterozygous or homozygous form in three Saudi *cblB* patients; the ethnicity of the fourth *cblB* patient carrying this mutation was not specified. The c.403G>A (p.A135T), c.571C>T (p.R191W) and c.656A>G (p.Y219C) mutations were seen together in 2 African American patients; no other patients in the panel had any of these mutations.

Genotype and phenotype correlation:

The majority of *cblB* patients in our collection came to medical attention within the first year of life, but clinical presentation ranged from the neonatal period to 14 years of age (Table 1). In addition, age at presentation was an incomplete reflection of disease severity; one patient in our panel died following a first acidotic crisis at the age of 12 years. The common c.556C>T mutation was usually associated with early onset; there were seven patients with known clinical course that were homozygous for c.556C>T, with age at presentation ranging from the neonatal period to 18 months (all but one presented in the first year). The c.197-1G>T mutation was seen in homozygous form in three patients; onset was at 3.8 and 14 years in two Saudi patients, but within the first month of life in a patient from North America. The African American *cblB* patients with the c.403G>A, c.571C>T and c.656A>G mutations both had late presentation (3 years and 8 years). The affected sister of one of these patients (not part of our panel) also carried the same mutations and presented at 14 years of age.

The effect of some of the most common mutations on biochemical function of fibroblasts was assessed. Incorporation of label from [^{14}C]propionate into cellular macromolecules, which is a measure of intact cell function of MCM, tended to be decreased in cell lines homozygous for the c.197-1G>T mutation compared to other *cblB* lines (Table 2); there was no response of propionate incorporation to supplementation of culture medium with OHCbl. Fibroblasts from cell lines carrying the c.700C>T (p.Q234X) mutation in either homozygous or heterozygous form had relatively high propionate incorporation with a vigorous response to supplementation with OHCbl. There

were no obvious correlations between specific mutations and AdoCbl synthesis in our assay system.

DISCUSSION

In the present study, sequence analysis of genomic DNA from a panel of *cblB* patient fibroblasts has been used to identify disease-causing mutations in the *MMAB* gene. Thirteen novel mutations were identified, bringing the total number of *MMAB* mutations identified in our laboratory to 19. Three additional mutations, c.558_559delGGinsC [18], c.287T>C (p.I96T) and c.584G>A (affecting a splice site) [19,20] have been reported. Of particular note, the previously described c.556C>T (p.R186W) mutation represented 33% of all disease causing alleles in our collection of 35 patients. This mutation has also been reported by other researchers [18-20]. In homozygous form it was associated with onset of symptoms typically within the first year of life; biochemical studies in patient fibroblasts were typical of the *cblB* class as a whole (table 2). Patients carrying this mutation were almost exclusively of European background, and shared a common haplotype across the *MMAB* gene, consistent with a single mutational event. However, this haplotype was observed frequently in controls, and was also observed in patients with other *MMAB* mutations, from other ethnic backgrounds. It is possible that the frequency of the c.556C>T mutation (a C to T transition at a CpG) is the result of more than one mutational event occurring on a common haplotype.

The recent determination of the crystal structure of a PduO class cobalamin adenosyltransferase from *T. acidophilum*, which is 32% identical to the human *MMAB* gene product, has provided insight into the structure of the active site of the enzyme [8]. The archaean enzyme functions as a homotrimer, with an active site formed by interaction of helix $\alpha 1$ of one subunit with the $\alpha 4$ helix of the adjacent subunit. A number of highly conserved residues located at the interface of two subunits, which are not involved in amino acid packing, were identified; it was suggested that these residues are involved in formation of the active site. A number of the *MMAB* mutations identified in this study (c.556C>T [p.R186W], c.557G>A [p.R186Q], c.568C>T [p.R190C], c.569G>A

[p.R190H], c.571C>T [p.R191W], c.572G>A [p.R191Q] and c.577G>A [p.E193K]) alter one of these highly conserved residues (Figure 1). Two of these mutations (p.R186W and p.E193K) have been shown to eliminate activity of the *P. acidophilum* adenosyltransferase *in vitro* [8]. The remaining missense mutations identified (c.521C>T [p.S174L], c.539C>G [p.S180W] and c.656A>G [p.Y219C]) also affect conserved residues within or close to the enzyme active site. Verification that these mutations affect enzyme function will require additional *in vitro* enzymological studies.

A single nonsense mutation (c.700C>T [p.Q234X]) was identified. This mutation occurs close to the 3'-end of the gene and does not affect any conserved portion of the protein. This mutation may affect stability of mRNA or of the protein product. Fibroblasts from patients with this mutation in homozygous or heterozygous form had higher levels of MCM function than most *cblB* fibroblasts (Table 2), although this does not appear to be correlated with any reduction in severity of clinical presentation.

The remaining mutations included mutations predicted to affect splicing of mRNA and duplications or deletions. Of note, the c.290G>A mutation is predicted to affect splicing between exons 3 and 4, and is also predicted to result in an amino acid change, p.G97E, affecting a conserved residue close to part of the active site of the protein product.

In two African American *cblB* patients (WG2487, WG2492), the same 3 sequence changes were detected within the *MMAB* gene. One change, c.571C>T (p.R191W), has been shown to abolish enzyme activity in the *T. acidophilum* adenosyltransferase. Of the remaining mutations, c.656A>G (p.Y219C) represents a nonconservative alteration at a conserved residue and may be a better candidate for a second disease-causing mutation than the c.403G>A (p.A135T) mutation, which was previously identified as a mutation in WG2492 [6]. Presumably, two of the sequence changes exist in cis in these patients.

Eleven of the nineteen mutations identified in this study are in exon 7 of the *MMAB* gene. These include two duplication mutations (c.563_577dupTGTGCCGCCGGGCCG and c.567_571dupCCGCC); this may reflect the presence of a GC-rich region that includes repetitive elements. Four of the 9 missense mutations identified in exon 7 represent C to T transitions at CpG sites (c.521C>T,

c.556C>T, c.568C>T and c.571C>T); cytosine residues at such sites have been recognized as particularly prone to mutation [21].

It was possible to identify some correlations between *MMAB* genotype and phenotype, both in clinical presentation and behavior of cultured fibroblasts. However, there does not appear to be any consistent correlation between the cellular and clinical phenotypes in *cblB* patients. Thus, the c.700C>T (p.Q234X) mutation in homozygous or heterozygous form was associated with a relatively mild defect in propionate incorporation in cultured fibroblasts, and a vigorous response of propionate incorporation to exogenous OHCbl (Table 2). However, this was not reflected in later age of onset of disease symptoms in patients carrying this mutation (Table 1). Similarly, the c.197-1G>T mutation was associated with a severe cellular phenotype, but patients homozygous for this mutation had relatively late ages at onset.

It has been noted that the *cblB* disorder generally has a more severe clinical course and is less responsive to therapy than the *cblA* disorder, although both disorders result in decreased AdoCbl synthesis. This has been attributed to the relative "leakiness" of *cblA* mutations [1]. It is notable that nearly all of the missense mutations that have been identified in *cblB* patients affect the putative active site of the adenosyltransferase (Figure 1). Thus, nearly all of the mutations identified in our collection are predicted to either cause formation of a truncated protein product (duplications and deletions, splice site mutations) or of a protein with a defective active site. This may reflect in part the high GC content and repetitive nature of exon 7, which encodes part of the enzyme active site. It is also possible that missense mutations affecting amino acid residues that occur outside of the active site result in a phenotype that is not recognized clinically as the *cblB* disorder; this might account for a portion of the patients with relatively mild methylmalonic aciduria that cannot currently be assigned to any of the known inborn errors of cobalamin metabolism.

ACKNOWLEDGEMENTS

We thank the clinicians who provided patient samples and clinical information, Nora Matiaszuk for complementation analysis, Gail Dunbar for growth of patient fibroblasts, and Vince Forgetta, Donna Sinnett, Corine Zotti and David Roquis for technical

assistance. This is a publication of the Hess B. and Diane Finestone Laboratory in the memory of Jacob and Jenny Finestone.

REFERENCES

[1]. D.S. Rosenblatt, D, W.A. Fenton, Inherited disorders of folate and cobalamin transport and metabolism, in: C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle (Eds), The metabolic & molecular bases of inherited disease, vol. III, McGraw-Hill, New York, 2001, pp 3897-3933.

[2] S.M. Matsui, M.J. Mahoney, L.E. Rosenberg (1983), The natural history of the inherited methylmalonic acidurias, N. Eng. J. Med. 308, 857-861.

[3] C.M. Dobson, T. Wai, D. Leclerc, H. Kadir, M. Narang, J.P. Lerner-Ellis, T.J. Hudson, D.S. Rosenblatt, R.A. Gravel (2002), Identification of the gene responsible for the cblB complementation group of vitamin B_{12}-dependent methylmalonic aciduria. Hum. Molec. Genet. 11, 3361-3369.

[4] W.A. Fenton, L.E. Rosenberg (1978), Genetic and biochemical analysis of human cobalamin mutants in cell culture. Ann. Rev. Genet. 12, 223-248.

[5] N. Korotkova, M.E. Lidstrom (2004). MeaB is a component of the methylmalomyl-CoA mutase complex required for protection of the enzyme from inactivation. J. Biol. Chem. 279, 13652-13658.

[6] C.M. Dobson, T. Wai, D. Leclerc, A. Wilson, X. Wu, C. Doré, T.J. Hudson, D.S. Rosenblatt, R.A. Gravel (2002), Identification of the gene responsible for the cblA complementation group of vitamin B_{12}-responsive methylmalonic acidemia based on analysis of prokaryotic gene arrangements, Proc. Natl. Acad. Sci.USA 99, 15554-15559.

[7] N.A. Leal, S.D. Park, P.E. Kima, T. Bobik (2003),. Identification of the human and bovine ATP:Cob(I)alamin adenosyltransferase cDNAs based on complementation of a bacterial mutant. J. Biol.Chem. 278, 9227-9234.

[8] V. Saridakis, A. Yakunin, X. Xu, P. Anandakumar, M. Pennycooke, J. Gu, F. Cheung, J.M. Lew, R. Sanishvili, A. Joachimiak, C.H. Arrowsmith, D. Christendat, A.M. Edwards (2004), The structural basis for methylmalonic aciduria. The crystal structure of archaeal ATP: Cobalamin adenosyltransferase. J. Biol. Chem. 279, 23646-23653.

[9] M. Yamanishi, T. Labunska, R. Banerjee (2005), Mirror "base-off" conformation of coenzyme B_{12} in human adenosyltransferase and its downstream target, methylmalonyl-CoA mutase. J. Am. Chem. Soc. 127, 526-527.

[10] T.A. Stich, M. Yamanishi, R. Banerjee, T.C. Brunold (2005), Spectroscopic evidence for the formation of a four-coordinate Co^{2+}cobalamin species upon binding to the human ATP:cobalamin adenosyltransferase. J. Am. Chem. Soc. 127, 7660-7661.

[11] M. Yamanishi, M. Vlasie, R. Banerjee (2005),. Adenosyltransferase: an enzyme and an escort for coenzyme B12? Trends Bioch. Sci 30, 304-308.

[12] D. Watkins (1998), Cobalamin metabolism in methionine-dependent human tumour and leukemia cell lines. Clin. Invest. Med. 21, 151-158.

[13] D. Watkins, N. Matiaszuk, D.S. Rosenblatt (2000), Complementation studies in the cblA class of inborn error of cobalamin metabolism: evidence for interallelic complementation and for a new complementation class (cblH). J. Med. Genet. 37, 510-513.

[14] B. Ewing, L. Hillier, M.C. Wendl, P. Green (1998), Base-calling of automated sequencer traces using Phred I. Accuracy assessment. Genome Res. 8, 175-185.

[15] D. Gordon, C. Abajian, P. Green (1998), Consed: A graphical tool for sequence finishing. Genome Res. 8, 195-202.

[16] The International HapMap Consortium (2005), A haplotype map of the human genome. Nature 437,1299-1320.

[17] J.C. Barrett, B. Fry, J. Maller, M.J. Daly, (2005), Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 21, 263-265.

[18] E. Pospisilova, L. Mrazova, D. Priby, P. Chrastina, R. Soucek, O. Martincova, S. Stastna, K. Halova, D. Kantarska, E. Gregova, M. Kosarova, I. Chodorova, H. Dvorakova, M. Elleder, J. Zeman (2005), Prenatal diagnosis in two families with methylmalonic acidemia due to cobalamin B deficiency. J. Inher. Med. Dis. 28, 84.

[19] M.A. Martinez, A. Rincón, L.R. Desviat, B. Merinero, M. Ugarte, B. Pérez (20050, Genetic analysis of three genes causing isolated methylmalonic acidemia: identification of 21 novel allelic variants. Molec. Genet. Metab. 84, 317-325.

[20] A. Rincón, B. Perez, B. Merinero, L.R. Desviat, M. Ugarte (2005), Clinical, biochemical and genetic analysis of five methylmalonic acidemia (MMA) patients belonging to the cblB complementation group. J. Inher. Med. Dis. 28, 82.

[21] A.R. Templeton, A.G. Clark, K.M. Weiss, D.A. Nickerson, E. Boerwinkle, C.F. Sing (2000), Recombinational and mutational hotspots within the human lipoprotein lipase gene. Am. J. Hum. Genet. 66, 69-83.

TABLES

Table 1. Mutation and biochemical analysis of patients with *cblB*

Patient Cell line	Sex/Race	Age of Onset	Mutation 1 cDNA	Mutation 1 Protein	Mutation 2 cDNA	Mutation 2 Protein	Prop-OH	Prop+OH	AdoCbl
WG2816	Unknown	<23 d	c.197-1G>T	r.spl?	c.197-1G>T	r.spl?	0.4	0.4	3.8
WG3176	M/W	3.8 y	c.197-1G>T	r.spl?	c.197-1G>T	r.spl?	0.5	0.4	N/D
WG3293	M/W	14 y	c.197-1G>T	r.spl?	c.197-1G>T	r.spl?	0.6	0.7	4.1
WG2523	F/W	7 m	c.197-1G>T	r.spl?	c.577G>A	p.E193K	0.2	0.5	1.2
WG3185	M/W	11 m	c.290G>A	p.G97E	c.568C>T	p.R190C	1.1	2.1	6.1
WG2350	M/W	3 d	c.291-1G>A	r.spl?	c.291-1G>A	r.spl?	0.5	0.5	1.6
WG2027	M/W	3 d	c.291-1G>A	r.spl?	c.556C>T	p.R186W	0.9	0.9	13.1
WG3332	F/B/W	4 d	c.291-1G>A	r.spl?	c.700C>T	p.Q234X	1.7	5.2	1.6
WG2235	F/W	12 y	c.521C>T	p.S174L	c.521C>T	p.S174L	4.4	5.4	3.6
WG2980	M/A	14 d	c.539C>G	p.S180W	c.568C>T	p.R190C	0.5	1.0	1.0
WG1185	Unknown	Unknown	c.556C>T	p.R186W	c.556C>T	p.R186W	1.1	ND	4.3
WG1493	M/W	3 d	c.556C>T	p.R186W	c.556C>T	p.R186W	0.3	0.4	2.2
WG2186	M/W	<1 w	c.556C>T	p.R186W	c.556C>T	p.R186W	1.0	1.1	2.5
WG1771	M/W	3 w	c.556C>T	p.R186W	c.556C>T	p.R186W	1.8	2.0	9.7
WG1792	M/W	3 m	c.556C>T	p.R186W	c.556C>T	p.R186W	3.4	4.1	2.1
WG1586	F/W	7 m	c.556C>T	p.R186W	c.556C>T	p.R186W	1.1	1.3	4.0
WG2846	F/W	11 m	c.556C>T	p.R186W	c.556C>T	p.R186W	0.8	1.0	1.1
WG3117	F/W	18 m	c.556C>T	p.R186W	c.556C>T	p.R186W	0.9	1.2	2.1
WG1879	M/W	5 m	c.556C>T	p.R186W	c.569G>A	p.R190H	1.0	1.5	3.4
WG2779	F/W	21 m	c.556C>T	p.R186W	c.572G>A	p.R191Q	1.2	2.3	2.5
WG2633[2]	F/W	7 d	c.556C>T	p.R186W	c.563_577dup	p.186_190dup	0.5	0.5	0.8
WG2268	M/W	2 d	c.556C>T	p.R186W	c.585-2A>C	r.spl?	0.7	0.6	3.3
WG2345	M/W	1 y	c.556C>T	p.R186W	c.700C>T	p.Q234X	2.8	5.2	6.0
WG2776	F/W	6.5 y	c.556C>T	p.R186W	c.700C>T	p.Q234X	2.8	4.2	2.3
WG3274	M/W	14 y	c.557G>A	p.R186Q	c.557G>A	p.R186Q	0.7	0.9	2.0
WG2147	M/B	5 d	c.563_577dup	p.186_190dup	c.563_577dup	p.186_190dup	0.8	1.0	3.3
WG3224	F/W	NB	c.567_571dup	p.R191PfsX25	c.567_571dup	p.R191PfsX25	0.5	0.4	1.3
WG3296	F/W	4 d	c.569G>A	p.R190H	c.569G>A	p.R190H	0.8	1.0	2.1
WG2492[1]	M/B	3 y	c.571C>T	p.R191W	c.656A>G	p.Y219C	2.0	3.6	6.5
WG2487[1]	F/B	8 y	c.571C>T	p.R191W	c.656A>G	p.Y219C	1.4	2.5	3.5
WG3230	M/B	3 d	c.585-2A>C	r.spl?	c.585-2A>C	r.spl?	0.9	0.7	0.4
WG2127	FW	4 d	c.656_659del	p.Y219SfsX4	c.700C>T	p.Q234X	2.1	4.1	2.3
WG1641	M/W	Infancy	c.700C>T	p.Q234X	c.700C>T	p.Q234X	2.8	4.8	4.2
WG0117	F/Unknown	11 m	c.700C>T	p.Q234X	c.700C>T	p.Q234X	2.4	ND	ND
WG1680	M/W	3 d	c.700C>T	p.Q234X	ND		2.8	5.0	2.8
***cblB*Avg**	NA	NA	NA	NA	NA	NA	1.4 ± 1.0	2.0 ± 1.7	3.4 ± 2.6
Controls	NA	NA	NA	NA	NA	NA	10.8 ± 3.7	10.9 ± 3.5	15.3 ± 6.7

Cell lines are arranged sequentially by the position of the first mutation from 5' to 3', and then by the age of onset.
F = Female, M = Male; W= White, B = Black, A = Asian.
NB = MMA detected on newborn screening; ND = Not Done; NA = Not Applicable
[1] Three sequence changes detected; third sequence change (c.403G>A [A135T]) not shown.
[2] The previously reported c.572_576delGGGCC mutation [6] could not be detected in this cell line.
Prop-OH: Incorporation of [^{14}C]propionate (nmols/mg prot/18h) without addition of OHCbl.
Prop+OH: Incorporation of [^{14}C]propionate (nmols/mg prot/18h) with addition of OHCbl.
AdoCbl: AdoCbl Synthesis (% of total intracellular Cbl). AdoCbl synthesis was within the control range in two patient fibroblast lines (WG1771 and WG2027); diagnosis of cblB in these patients was established by somatic cell complementation analysis.

Table 2. Effect of genotype on biochemical parameters in *cblB* patients

Genotype	Propionate Incorporation (nmols/mg prot/18h)		AdoCbl (% of total)
	Without OHCbl	With OHCbl	
cblB (n=35)	1.4 ± 1.0	2.0 ± 1.7	3.4 ± 2.6
c.556C>T/c.556C>T (n=8)	1.3 ± 0.9	1.6 ± 1.2	3.5 ± 2.6
c.197-1G>T/c.197-1G>A (n=3)	0.4; 0.5; 0.6	0.4; 0.4; 0.7	3.8; 4.1
c.700C>T/c.700C>T (n=2)	2.4; 2.8	4.8	4.2
c.700C>T/c.556C>T (n=3)	2.8; 2.8; 2.8	4.8; 5.2; 4.2	4.2; 6.0; 2.5
c.700C>T/other* (n=6)	2.5 ± 0.5	4.8 ± 0.5	3.2 ± 1.6

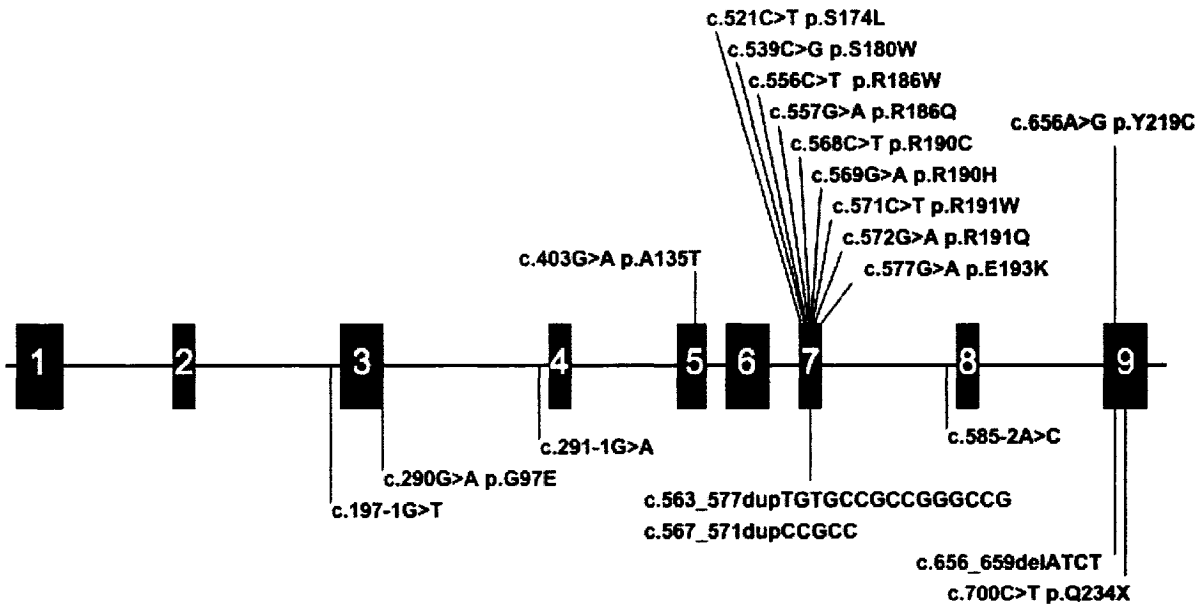
Values are means ± SD; when the number of patients was three or less, individual values are given.

* Compound heterozygous patients carrying c.700C>T plus any other mutation.

Figure Legend

Figure 1

Mutations identified in the *MMAB* gene. The gene structure of the *MMAB* gene according to reference sequence NM_052845.2 is shown. Missense mutations are shown above the plane of the gene; duplications, deletions, splice-site mutations, and a premature termination codon mutation are shown below the plane of the gene.



CHAPTER 4.0: The Molecular basis of *cblC* disease

The genetic mapping of *cblC* disease to chromosome 1 was initially carried out by Janet Atkinson as part of her Master's thesis at the University of Toronto (208). Data generated from this work were used as the basis for further investigation of the chromosomal interval. Sibling pair analysis, homozygosity mapping and haplotype analyses were subsequently used to further refine the chromosomal interval and to identify the gene for *cblC* disease. These methods and results are described below.

4.0.1 Linkage Analysis

In 2002 Atkinson (*et al.*) reported the genetic mapping of *cblC* to the short arm of chromosome 1 (209) and in 2003 putative linkage of *cblC* was identified in the critical interval of chromosome region 1p32-34 (208). DNA was collected from a total of forty-four individuals including eleven families and twelve *cblC* patients. Six families reported consanguinity (five 1^{st} cousin marriages and one 2^{nd} cousin marriage). Informed consent was obtained from the families recruited and *cblC* disease was confirmed in all cases by complementation analysis in the laboratory of David S. Rosenblatt, MD.

A 10 cM genome scan using 306 microsatellite markers (di-, tri-, or tetranucleotide repeats) with an average heterozygosity value of 0.77 was performed on seven families in the lab of Dennis Bulman (Ottawa Health Research Institute). These markers were from the Cooperative Human Linkage Center (CHLC) Human Screening Set, version 8.0, and from the Marshfield database (210,211). PCRs were multiplexed with two or three marker sets, visualized on a 6% sequencing gel on a LI-COR Near-IR Fluorescence Automated DNA Sequencer and RFLPscan software was used for analysis (Scanalytics, Billerica, MA). Six two-point lod scores of >+1.0 were achieved with two-point linkage analysis (recombination fractions of 0-0.4): D1S2864, D1S2134, D1S1609, D2S441, D5S2114, and D10S1423. Additional markers obtained from publicly available genetic maps including Marshfield and DeCODE Genetics were used to evaluate regions of the genome where potential linkage was detected. The markers were selected based on a location of 1-5 cM from the original marker set and on heterozygosity values of >0.75. If markers were not available they were identified by analysis of the public DNA

sequence database, tested for unique sequence and for heterozygosity to fill in spaces in the genetic map.

SLINK was used to calculate the power to test linkage (performed by Andrew Paterson). An average lod score of 4.73 and a maximum lod score of 5.63 at $\theta = 0$ was observed indicating the ability to detect linkage. Two-point lod scores were calculated with the program MLINK (212,213) at recombination fractions of 0, 0.01, 0.05, 0.1, 0.2, and 0.4. Each family was analyzed separately with an overall lod score calculated from the sum of individual familial lod scores. The parameters used in MLINK included the assumption of no sex difference in recombination rates, autosomal recessive inheritance with 100% penetrance and a disease allele frequency of 0.002, based on an estimated disease frequency of 1 in 1 000 000. Five loci were eliminated as loci linked to the disease by analysis of flanking markers and because they produced two-point lod scores between –5.0 and –2.0 at a $\theta = 0$. Linkage was established to chromosome 1 by analysis of markers flanking D1S2134. The loci producing positive lod scores spanned a genetic interval of 16 cM. A two-point lod score of 3.01 was determined for D1S2134 and a maximum two-point lod score of 4.04 was achieved 2 cM distal to marker D1S211.

Multipoint linkage analysis was performed using GENEHUNTER 2.1 (214,215). The parameters used were the same as those used for MLINK with marker distance expressed in cM obtained from the Marshfield sex averaged map. The multipoint analysis confirmed the two-point analysis. The broad interval from D1S3721 to D1S427 produced lod scores of 7.16 over an area of ~4.7 cM. Haplotypes were analyzed and a shared region of homozygosity for the six consanguineous families extended ~3 cM (D1S3721 to D1S6542). Two individuals were heterozygous at each of the boundaries slightly reducing the defined region.

The transmission disequilibrium test (TDT) was used to measure the frequency of transmission of alleles from heterozygous parents to affected children to determine if the frequency deviated from 50% (216). A χ^2 test was used to assess the significance of linkage disequilibrium. The TDT generated a χ^2 value of 8.00 ($p = 0.005$) for allele #6 of marker D1S2134. This allele was consistently transmitted eight times to affected offspring from heterozygous parents. No other marker in the critical region showed significant allelic association. This allele had a frequency of 0.3077 in the normal

population, as compared to a patient frequency of 0.4872. The order of markers in this region was verified by radiation hybrid mapping and aligned according to genetic and physical maps. Two Acadian individuals were later reported as sharing a common haplotype between markers JA7079_1 and D1S2134 (221).

4.0.2 Homozygosity Mapping

In 2002, homozygosity mapping was carried out by Jamie C. Tirone, using microsatellite markers spanning the interval containing marker D1S2134. Homozygosity mapping is a useful way to map human recessive traits with the DNA of patients from consanguineous marriages (217). Early studies on inborn errors by Garrod noted a high proportion of patients with alkaptonuria were progeny of consanguineous marriages (218). Bateson supplied the Mendelian explanation and subsequent studies demonstrated that the rarer the disease, the more pronounced the effect (217,219).

This gene mapping strategy relies on the fact that children of consanguineous marriages are expected to share more of their genome than would be expected by chance. Homozygous segments can extend for many centimorgans (cM) (up to 28 cM surrounding the disease gene) and it is expected that affected individuals will be homozygous by descent at the disease locus. Searching for areas that are consistently homozygous by descent in different families is a powerful strategy to map human recessive genes (217).

If a child is affected then homozygosity by descent at the disease locus is expected and the probability is: $\alpha = Fq/[Fq + (1 - F)q^2]$; where F is the coefficient of inbreeding which is the fraction of the child's genome expected to be homozygous by descent (For siblings, first cousin and second-cousin marriages: F = ¼, 1/16, 1/64, respectively); where q is the frequency of a disease allele in a population in Hardy-Weinberg equilibrium. Note that Fq of all affected individuals will be homozygous by descent at the disease locus, while $(1 - F)\ q^2$ will not be homozygous by descent at the disease locus but will be affected due to random meeting of disease alleles. If q is small compared to F, then α is very close to 1. For a disease locus homozygous by descent $\alpha \sim 1$ whereas unlinked regions have a probability of F ($<< 1$). The use of highly polymorphic markers is essential

for this type of study to increase the likelihood that homozygosity for any allele implies homozygosity by descent. In this case studying only 3 affected individuals from a first-cousin marriage (F = 1/16) would yield an odds ratio of 16^3:1, which is over the threshold of 1000:1 required for proving linkage (220). Multilocus linkage analysis of multiple loci in a pedigree can be calculated using programs that compute multilocus likelihoods using efficient algorithms that can perform this anlaysis. It is important in this type of study that adequate marker density be addressed because of recombination opportunities as well as the degree of inbreeding should be taken into consideration. For example, the disease allele must be as low as $q < 0.02$ (frequency of the cystic fibrosis allele in the U.S. (217)) for progeny of first cousin marriages to ensure adequate statistical significance and $q < 0.008$ for progeny of second cousins.

Although the surrounding region of homozygosity by descent is fairly large in any given child (median length of ~28 cM for affected progeny of 1st cousin marriages), the search for a given gene may be confined to the overlap of these regions (if n = the number of affected first-cousin progeny studied then the overlap may be: ~28 cM/n (217).

As partly described in the published work from this chapter, Jamie Tirone genotyped 180 *cblC* patients including 22 individuals from consanguineous matings, three pairs of affected siblings and 50 unrelated controls for 26 microsatellite markers in the interval between markers D1S3721-D1S2134, a distance of 6.6 Mb and containing ~189 genes (Supplementary Table 1. (221)). The three pairs of siblings were concordant for identical alleles across this entire region confirming the authenticity of the orginal linkage study.

The controls selected were a cohort of individuals with inborn errors of metabolism other than *cblC* disease (including *mut*, *cblA*, and *cblB*), to account for bias of ascertainment of individual samples as well as for background homozygosity due to increased probability of inbreeding for rare recessively inherited disease. Patient genotype data were analyzed for presence of overlapping regions of homozygosity, haplotype similarity, and marker heterogeneity, as compared to controls.

Two non-adjacent markers were homozygous by descent in all 22 individuals from consanguineous matings. One homozygous region surrounding marker D1S421 between markers D1S2713 and D1S2802 was identified within this 6.6 Mb region. Ten

genes selected in the region surrounding this marker were screened for mutations in at least eight patients with *cblC* disease. Numerous heterozygote SNPs were detected in several genes in three individuals from consanguineous matings suggesting that these regions had been broken down by recombination events and eliminating this region as being inherited homozygous by descent. This region was concluded to be unrelated to the disease locus, a known pitfall of homozygosity mapping (222). Extended regions of homozygosity have been suggested to be quite common (223) and uninformative markers are also potential pitfalls of this method.

The other marker that showed homozygosity in all consanguineous individuals tested was JA2001. Genotyping was carried out for confirmation of homozygosity in all consanguineous patient samples for putative marker JA2001. The region surrounding this marker was further investigated by selectively screening 17 candidate genes for mutations. Both Marker D1S421 & JA2001 were later determined to be within the haplotype region identified by Atkinson *et al* in two Acadian individuals. The candidate gene region encompassed markers flanking D1S421 & JA2001, markers D1S2713 & D1S2797 and including 35 genes. During the screening of candidate genes in this region, a second haplotype shared by two affected patients, one Italian and one Iranian, was identified and narrowed the candidate region to 911 kb bounded by D1S2677 and rs2292487. Marker D1S3175 was excluded from this haplotype as marker heterogeneity, genotyping error or marker slippage was suspected. Remaining details on the gene identification are as described in Lerner-Ellis et al, Nat. Genet. 2006, in Chapter 4.1.

4.0.3 Candidate genes - Identification of mutations and polymorphisms

Examination of expressed sequence tags (EST) databases was used to construct a composite human cDNA for genes with known or unknown functions identified within the candidate regions surrounding homozygous markers D1S421 and JA2001. ESTs in other mammals were used to support the authenticity of the composite cDNA and examined for the presence of conservation of intron/exon boundaries and gene structure. Candidate genes were selected based on function, conservation and expression profiles. The *cblC* gene was hypothesized to have reductase activity or ß-ligand transferase activity

or other vitamin B_{12} related functions (108). Thus, domains consistent with these functions were sought. Within the candidate region, eight genes that were known to have reductase activity were screened for mutations in eight consanguineous *cblC* patients. No apparent mutations were identified.

Genes within candidate regions were examined for additional properties using standard bioinformatic tools including protein-protein (blastp) and conserved domain (rpsblast) databases available through the National Center for Biotechnology and Information (NCBI). The *cblC* gene was hypothesized to localize to the cytoplasm; PSORTII was used to analyze specific compartment targeting sequences. Identification of domains with cobalamin related functions in bacteria has been useful in characterizing the functional properties of all of the genes that have been identified to date in this metabolic pathway. Cobalamin processing is not carried out in yeast, and so candidate genes should not have orthologues present in the yeast genome. The *cblC* protein was expected to be ubiquitously expressed at low levels as is seen with most other genes in this pathway; perhaps with higher levels in liver and muscle as seen with the genes responsible for *cblA* and *cblB* diseases. Expression data from publicly available GNF Atlas databases and others was used to determine expression profiles of candidate genes.

4.0.4 Mutation Screening

The patient samples used for mutation screening from cDNA or gDNA were 12 samples from *cblC* patients from consanguineous matings and 16 *cblC* individuals from marriages where no consanguinity was reported, as well as three controls. RNA (cDNA) and gDNA were isolated from patient fibroblast cell lines, and were screened for mutations by automated sequencing of specific candidate genes. Note that not all of these samples were examined for each candidate gene and a combination of sequencing from cDNA and gDNA was employed; especially when ESTs suggested that a candidate gene had alternative splice site variation. The identification of null mutations in patient DNA, such as those caused by large insertions or deletions or other changes resulting in truncation of the protein product would provide strong evidence that mutations in the candidate gene is the molecular basis of *cblC* disease.

From the mutation-screening panel above, patients from consanguineous matings were the most informative with sequence changes in the regions suggesting either homozygosity by descent or no homozygosity by descent. From our cohort, one turkish, two Lebanese, one Iranian, three Pakistani, two Canadian, one Cajun, one Saudi Arabian, and one Hispanic individuals were used for homozygosity mapping. The range of different ethnic groups was selected in order to increase the probability of picking up different mutations and to decrease the probability of missing a mutation. Different ethnic groups are likely to carry different ancestral mutations, as has been noted in many other recessively inherited diseases including other diseases of cobalamin metabolism including *cblA*, *cblB*, and *mut* disease (224,225,226), although this may not always be the case. From the cohort of individuals with no report of consanguinity, six Mexican or Hispanic individuals, three Italian, four Caucasian, one Danish and two Asiatic Indians were selected. Ultimately, a common haplotype was identified in one individual from the consanguineous panel and one from the panel with no report of consanguinity and led to the identification of the gene responsible for *cblC* disease.

4.1 Identification of the gene responsible for methylmalonic aciduria and homocystinuria, cblC type

Jordan P. Lerner-Ellis[1,2], Jamie C. Tirone[1,2], Peter D. Pawelek[3], Carole Doré[4], Janet L. Atkinson[5], David Watkins[1,2], Chantal F. Morel[1,2], T. Mary Fujiwara[1,2], Emily Moras[1,2], Angela R. Hosack[2], Gail V. Dunbar[2], Hana Antonicka[1,6], Vince Forgetta[4], C. Melissa Dobson[7], Daniel Leclerc[1,2], Roy A. Gravel[7], Eric A. Shoubridge[1,6], James W. Coulton[3], Pierre Lepage[4], Johanna M. Rommens[5], Kenneth Morgan[1,2], David S. Rosenblatt[1,2]

[1]Department of Human Genetics, McGill University, Montreal, Quebec, Canada, H3G 1A4.

[2]Division of Medical Genetics, McGill University Health Centre, Montreal, Quebec, Canada, H3G 1A4. [3]Department of Microbiology and Immunology, McGill University, Montreal, Quebec, Canada, H3A 2B4.

[4]McGill University and Genome Quebec Innovation Centre, Montreal, Quebec, Canada.

[5]Department of Molecular and Medical Genetics, University of Toronto and Program in Genetics and Genomic Biology, The Hospital for Sick Children, Toronto, Ontario, Canada, M5G 1X8.

[6]Montreal Neurological Institute, McGill University, Montreal, Quebec, Canada, H3A 2B4.

[7]Department of Biochemistry and Molecular Biology, University of Calgary, Calgary, Alberta, Canada T2N 4N1.

Correspondence should be addressed to D. S. R. (david.rosenblatt@mcgill.ca).**Published: Nature Genetics. 2006 Jan;38(1):93-100. Epub 2005 Nov 27.**

ARTICLE

Methylmalonic aciduria and homocystinuria, cblC type (OMIM 277400), is the most common inborn error of vitamin B_{12} (cobalamin, Cbl) metabolism, with about 250 known cases. Patients have developmental, hematological, neurological, metabolic, ophthalmologic and dermatologic findings[1]. Although considered a disease of infancy or childhood, some patients develop symptoms in adulthood[2]. The cblC locus was mapped to chromosome region 1p by linkage analysis[3]. We report refinement of the chromosomal interval using homozygosity mapping and haplotype analyses, and identification of the MMACHC gene. In 204 patients, 42 different mutations were identified, many consistent with a loss of function of the protein product. One mutation, c.271dupA, accounted for 40% of all disease alleles. Transduction of wild-type MMACHC into immortalized cblC patient cell lines corrected the cellular phenotype. Molecular modeling predicts that the C-terminal region of the gene product folds similarly to TonB, a bacterial protein involved in energy transduction for vitamin B_{12} uptake.

Hyperhomocysteinemia has been correlated with increasing numbers of medical disorders including birth defects, cardiovascular disease and stroke, psychiatric disease, dementia and cancer[4]. Cbl is an important cofactor in homocysteine metabolism and in branched-chain amino acid and odd-chain fatty acid catabolism. It is synthesized by bacteria and not found in plants; thus, intake of animal products or supplementation with Cbl is required to prevent deficiency. In mammalian cells, exogenous Cbl is converted to two active coenzyme derivatives: methylcobalamin (MeCbl) is required for activity of the cytoplasmic enzyme methionine synthase that converts homocysteine to methionine; adenosylcobalamin (AdoCbl) is required for activity of the mitochondrial enzyme methylmalonyl CoA mutase that converts L-methylmalonyl-CoA to succinyl-CoA.

Three affected sibling pairs were concordant for 26 microsatellite markers (Supplementary Table 1) spanning the previously described candidate region between markers D1S3721 and D1S2134 (2.9 cM, 6.6 Mb)[3](Atkinson JL, Paterson A, Renaud D, Clark JTR, Wilcken B, Bulman D, Rommens JM. (2002) Genetic mapping of Cobalamin C deficiency: putative linkage to 1p. Am J Hum Genet suppl: 71:452), supporting gene assignment to this interval (Fig. 1a). Haplotype analysis reduced this candidate region to a

3.8 Mb interval bounded by JA7079_1 and D1S2134, based on the smallest shared haplotype segment in two Acadian patients (haplotype A; Fig. 1a).

Twenty-two consanguineous cblC patients were homozygous for two nonadjacent markers, D1S421 and JA2001; this contrasted with 35% and 36% homozygosity, respectively, in 48 controls. Candidate genes were selected in the intervals surrounding these markers. Twenty-four SNPs identified during sequencing of candidate genes in the interval between D1S3175 and D1S2797, were homozygous in eight patients (four with reported consanguinity and four without). A shared region of homozygosity was identified between D1S3175 and SNP rs2292487 in two of these patients, reducing the candidate interval to 686 kb. No apparent causal mutations were detected in nine genes within this region in eight cblC patients. When marker D1S3175 was reevaluated and excluded from the haplotype, a longer homozygous segment of 911 kb, bounded by D1S2677 and rs2292487 (haplotype B; Fig. 1a), was identified that encompassed six additional genes. Four sequence changes were detected in a gene identified on the basis of EST AL080062.1 (NM_015506.1) (Fig. 1b, 1c). Sequence changes included two duplications (c.271dupA and c.450_479dup30) and two mutations creating premature termination codons (c.331C>T and c.394C>T). None of these sequence changes were detected in 105 controls. The putative cblC gene was sequenced in members of four families segregating cblC, and in all cases, the segregation of mutations was consistent with an autosomal recessive mode of inheritance (Supplementary Fig. 1). These data strongly indicate that this is the gene mutated in cblC patients. We have designated MMACHC as the gene symbol for methylmalonic aciduria cblC type with homocystinuria.

Three mRNA species of ~1.9, 3.0, and 5.4 kb were detected by Northern blot analysis using a multiple human tissue Northern blot (Clontech) and a 600 bp probe directed against coding positions c.65 to c.664 of MMACHC (data not shown). The 3.0 kb message predominated followed by the 5.4 and 1.9 kb messages. The gene appeared to be expressed in most tissues but higher levels of all three transcripts were detected by Northern blot in fetal liver with lower levels in spleen, lymph node, thymus and bone marrow. The messages were not readily detected in peripheral blood leukocytes (Supplementary Note).

The cellular cblC phenotype was complemented in two immortalized cblC fibroblast cell lines infected with wild-type MMACHC cDNA in a pLXSH retroviral vector. Function of both methionine synthase and of methylmalonyl CoA mutase, measured by the incorporation of label from 5-[^{14}C]methyltetrahydrofolate and [^{14}C]propionate into cellular macromolecules, was restored to control levels, or above, in infected cblC fibroblasts (Fig. 2a, 2b). Normal synthesis of both MeCbl and AdoCbl from exogenous cyanocobalamin (CNCbl) was also restored in infected cblC fibroblasts (Fig. 2c). The intracellular level of CNCbl, the stable nonphysiological Cbl derivative administered to cells, was decreased in both cblC and wild-type fibroblasts transduced with MMACHC (Fig. 2c), suggesting that CNCbl was recruited into cellular metabolism more effectively. Clinically, cblC patients have responded better to therapy with hydroxylcobalamin (OHCbl) than CNCbl[5]; cblC fibroblasts in culture utilize OHCbl more efficiently than CNCbl[6].

The ORF and flanking exon sequences of the MMACHC gene, and the non-coding portion of exon 4 and exon 5 (Fig. 1b), were sequenced from gDNA. Two putative mutations were identified in each of 185 of 204 patients; one mutation was identified in 5 patients; and in 14 patients no causal mutation was identified. Forty-two different mutations were identified. There were 13 missense mutations, 12 nonsense mutations, 9 deletions that would result in frameshifts, 1 inframe deletion, 1 duplication with frameshift, 1 duplication without frameshift, 2 mutations affecting the initiation codon, and 3 potential splice-site mutations (Table 1; Fig. 1c); none of these was found in 105 controls. The most common mutation, c.271dupA, occurred in 40% of all disease alleles. Seven mutations (c.217C>T, c.331C>T, c.394C>T, c.457C>T, c.481C>T, c.482G>A,

c.616C>T) involved C>T transitions at cytosine residues adjacent to guanine, a potential mutation hotspot[7]. Four mutations may be due to slippage during DNA replication: c.388_390delTAC (TAC x3), c.435_436delAT (AT x2), c.468_469delCT (flanked by GG), and c.547_548delGT (GT x2).

PHASE v2.1.1[8,9]was used to estimate the frequencies of the most likely haplotypes constructed from eight sequence variants identified within and flanking MMACHC in 54 CEPH individuals. Four common haplotypes were among the 19 different haplotypes inferred from CEPH individuals (Fig. 3a). Haplotypes for 99 cblC patients homozygous for one of 17 different MMACHC mutations indicated that most mutations arose on one of the three most common haplotypes (Fig. 3b).

The c.271dupA mutation was seen in patients from several different ethnic groups, but primarily European, and was found predominantly on haplotype #2 (Fig. 3b). Eight of 102 disease alleles carrying c.271dupA differed from this haplotype at a single nucleotide (c.1-302T>G, c.1-50A>G, or c.800G>A). These data are consistent with a single mutational event, followed by mutation or recombination modifying the ancestral haplotype. Analysis of microsatellite markers directly flanking MMACHC in 43 homozygous c.271dupA patients, including markers D1S2677 (179 kb telomeric to MMACHC) and D1S3175 (47 kb centromeric to MMACHC), is consistent with this hypothesis (data not shown). The c.800G>A (p.R267Q) sequence change was observed only on three chromosomes carrying the c.271dupA mutation and segregated with c.271dupA in one family.

Seven mutations showed clustering by ethnicity. Three of these (c.3G>A, c.331C>T and c.394C>T) were each found on two different haplotypes and may have arisen independently at least twice (Fig. 3b). Of these, c.331C>T and c.394C>T occur at potential CG hotspots. The 11 patients homozygous for c.394C>T, were Indian, Pakistani, or Middle Eastern. Five c.331C>T haplotype #1 homozygotes were French Canadian or Acadian, whereas two other homozygotes with a different haplotype were European. Three patients with c.3G>A on haplotype #2 were Italian or Northern European; the patient with c.3G>A on haplotype #3 was also European. Three patients with c.440G>A were Native American; four patients with c.547_548delGT were Lebanese; three patients

with c.608G>A were Hispanic; and the five patients with c.609G>A were East Asian (Fig. 3b).

We compared genotype and phenotype in patients carrying the most common MMACHC mutations: c.271dupA (p.R91KfsX14) and c.331C>T (p.R111X) were associated with early-onset disease, while c.394C>T (p.R132X) was associated with late-onset disease (Table 2). In contrast to patients with early-onset disease, the late-onset group was characterized by acute neurological deterioration without systemic symptoms[2]. Differences in RNA stability or residual function of the protein product are predicted to cause differences in phenotypes[10].

The MMACHC protein is not a member of any previously identified gene family. It is well conserved among mammals. However, the C-terminal end does not appear to be conserved in eukaryotes outside Mammalia, and no homologous protein was identified in prokaryotes (Supplementary Note). Motifs were identified in MMACHC that are homologous to motifs in bacterial genes with vitamin B_{12}-related functions. Residues 118–138 of MMACHC had 52% amino acid similarity (52% identity) to residues 629–642 in methylmalonyl CoA mutase from Streptomyces avermitilis (NP_823216.1), which is part of its Cbl-binding domain; this includes sequence with identity to a portion of the canonical Cbl-binding motif (^{122}HXXG-X_{29}-GG)[11], which was conserved in all species analyzed (Fig. 4a). Residues spanning 181–282 in MMACHC had similarity to various TonB proteins from Gram-negative bacteria. The highest degree of similarity was to TonB from Salmonella typhimurium: MMACHC amino acid residues 242–280 had a similarity of 51% (41% identity) to residues 119–156 of S. typhimurium TonB (P25945).

We aligned the primary sequence of MMACHC residues 185–282 to E. coli TonB residues 152–239, for which a three-dimensional structure is known[12-14], using the computer program Modeller[15] to generate a three-dimensional model structure of the MMACHC C-terminal domain. The overall structure of the model (Fig. 4b) is homologous to the recently-solved three-dimensional structure of the monomeric C-terminal domain of E. coli TonB[14]. It conforms to acceptable stoichiometric parameters. Ramachandran analysis indicated that no residues occurred within disallowed regions, with 80% of residues occurring within the core regions, 17% within allowed regions, and 3% within generously allowed regions. No bad contacts were found within the model.

PROCHECK[16] analysis revealed that the MMACHC model had an overall average G factor of -0.360, above the threshold value (-0.500) that reflects a protein fold with acceptable stoichiometry. The three-dimensional structures of the MMACHC model and the monomeric C-terminal domain of TonB were aligned by least-squares methods using the program LSQMAN[17]. Visual inspection of the superimposed Cα traces of the two proteins demonstrated that the MMACHC model shared a high degree of similarity to the TonB fold (Fig. 4c). Although the amino acid sequence identity across the region of superposition is only 14%, the MMACHC model and the C-terminal domain of TonB superimposed with a normalized root-mean-square distance (rmsd) value of 0.878 Å.

TonB is involved in transducing energy generated from the proton motive force to the transport of iron and vitamin B_{12} across the outer bacterial membrane. We hypothesize that the C-terminal domain of the MMACHC protein functions in a manner similar to the TonB C-terminal domain of E. coli. In this case, MMACHC would make direct contact with a cognate protein partner to facilitate conformational changes necessary for Cbl metabolism. The only region of the MMACHC model that deviates significantly from the TonB structure occurs between MMACHC residues 198–203. In the alignment of MMACHC with E. coli TonB, these residues are present as an insertion relative to the E. coli TonB sequence. They cause the formation of a 5-residue loop in the model that deviates from the protein backbone of a superimposed TonB structure (Fig. 4c, arrow). Significantly, the corresponding TonB residue in E. coli found at the position of maximal structural deviation is Q160, which has been previously shown to interact with the highly conserved Ton box of TonB-dependent outer-membrane receptors[14,18,19]. Variability in this region is consistent with the direct interaction of the MMACHC protein with a cognate protein partner through a motif different than the Ton box (Supplementary Note).

Currently, eight different defects in cellular Cbl metabolism have been defined (complementation groups cblA–cblH)[1]. The cblC, cblD, and cblF disorders result in combined methylmalonic aciduria and homocystinuria. Fibroblasts from cblF patients accumulate unmetabolized free CNCbl in lysosomes, and a defect in lysosomal Cbl transport in cblF was hypothesized[20]. Both cblC and cblD fibroblast cells demonstrate normal receptor-mediated endocytosis of the transcobalaminII(TCII)-Cbl complex and

normal intralysosomal hydrolysis of TCII, but do not accumulate Cbl in the lysosome. The defects in both cblC and cblD cells have been postulated to affect a step subsequent to cellular Cbl uptake, but prior to the synthesis of AdoCbl and MeCbl and to the binding of the Cbl coenzymes to their respective apoproteins. It has been suggested that reduction of the central cobalt atom of Cbl and removal of the upper axial ligand are necessary steps[21]. It is possible that the MMACHC gene product plays a role, directly or indirectly, in removal of the upper axial ligand and/or reduction of Cbl, and this is a challenge for future studies. MMACHC may be involved in the binding and intracellular trafficking of Cbl. Further studies on co-localization and a search for novel binding partners may help us to better understand the early steps of cellular vitamin B_{12} metabolism.

METHODS

Cell culture studies

For the past 22 years cultured fibroblasts from patients suspected of having an inborn error of Cbl metabolism have been sent to our laboratory for clinical diagnosis and characterization. The unique identification codes assigned to cell lines by the Repository for Mutant Human Cell Strains at the Montreal Children's Hospital (http://www.cellbank.mcgill.ca) were used. Referring physicians indicated whether cell lines should not be used to pursue research into the origin of these diseases. The procedure was approved by the Royal Victoria Hospital Research Ethics Board. If physicians were contacted to request samples of DNA or cell lines from additional family members, informed consent was obtained from the patient or his/her family.

Cell culture studies were carried out on 215 cblC patients (120 males; 94 females; 1 unknown). This collection of cblC cell lines included 10 pairs of affected siblings and 1 affected cousin-pair; 18 affected individuals were reported to be offspring of first-cousin marriages, 1 from a second-cousin marriage, and 5 had parents that were more distantly related. Only patients considered to be unrelated were included in the mutation analysis tabulations. This included 204 cblC patients (118 males; 85 females; 1 unknown). Of these, 48% were of European ancestry, 17% Hispanic, 17% Asian, and 19% of other or unknown ethnicity.

Fibroblasts from all patients in this collection had decreased incorporation of label from [^{14}C]propionate and 5-[^{14}C]methyltetrahydrofolate into cellular macromolecules (measuring function of methylmalonylCoA mutase and methionine synthase, respectively) and decreased synthesis of both AdoCbl and MeCbl from exogenous [^{57}Co]CNCbl. In all cases, diagnosis was confirmed by complementation analysis[23,24].

Genotyping Studies

DNA was extracted from patient and control fibroblasts using the Qiagen gDNA extraction kit for cultured cells. The DNA from 50 unrelated patients with inborn errors of vitamin B_{12} metabolism different than cblC was used as a comparison group for homozygosity mapping to control for bias of ascertainment of patient samples. Initial studies utilized a panel of 26 microsatellite markers selected from deCODE Genetics and Marshfield genetic maps (Supplementary Table 1). Markers were selected within and flanking the interval between markers D1S3721 and D1S2134 on chromosome 1p and were selected on the basis of length and purity of the repeat unit. Polymerase chain reactions (PCRs) performed in ABI 9700 thermal cyclers (Applied Biosystems) using fluorescently labeled primers to assay microsatellites gave products that were visualized with automated sequencers (ABI 3700 & 3730). An internal size standard (GENESCAN 400-Rox for ABI 3700 and GENESCAN 500-Liz for ABI 3730, Applied Biosystems) ensured accurate sizing. Electropherograms were analyzed with Genotyper v.3.6 and GeneMapper v.3.7 software (Applied Biosystems) for generating allele sizes. Mendelian inheritance testing and binning of alleles were done using PedmanagerW software, a modified version of Pedmanager.
(http://www.broad.mit.edu/ftp/distribution/software/pedmanager/).

Sequencing Studies

DNA was amplified by PCR from gDNA or cDNA. For sequencing analysis, 215 cblC patients, 55 unrelated CEPH controls, and 50 controls (patients with other inborn errors of vitamin B_{12} metabolism) were included. Of the 105 controls, 62% were of European ancestry, 24% Hispanic, 12% Asian and 2% of unknown ethnicity. Primers

used to amplify MMACHC are listed in Supplementary Table 2. Primers were designed using the Primer 3.0 software (www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). cDNA was prepared by whole RNA extraction from fibroblast cultures using Trizol reagent (Molecular Research Center) and mRNA was reverse transcribed with MMLV reverse transcriptase (USB) or superscript reverse transcriptase (Qiagen) and poly dT oligonucleotides (Amersham). PCR products were purified with Montage PCR_{96} filter plates (Millipore). Sequencing reactions (10 µl) contained 2 µl purified PCR product, 1 µl BigDye Terminator Cycle Sequencing Version 3.1 (Applied Biosystems), 1.5 µl of 5x buffer, 5 µl H_2O, and 0.5 µl (1 µM final) of sense or antisense primer. Products were analyzed on an ABI 3730x1 DNA Analyzer (Applied Biosystems). Sequencing files were processed using Sequence Analysis software (Applied Biosystems) and were assembled and analyzed using the Phred/Phrap/Consed System[25,26].

Haplotype Analysis

SNPs and deletion/insertion polymorphisms (DIPs) identified by sequencing of the MMACHC gene were used to construct haplotypes. The frequencies of haplotypes inferred from 54 CEPH controls were obtained using PHASE v2.1.1[8,9]. Haplotypes of cblC patients homozygous for MMACHC mutations could be deduced since they were heterozygous for at most one sequence variant.

Expression of Human MMACHC cDNA in patient cells

Wild-type MMACHC cDNA was generated by RT-PCR from total RNA extracted from normal human fibroblasts and confirmed by sequencing. This product was cloned into a Gateway expression vector system (Invitrogen) using the pENTR-TOPO directional cloning kit as recommended by the manufacturer, then subcloned into a pLXSH retroviral vector[27] containing Gateway cloning adaptors (Invitrogen). Fibroblast cell lines from two cblC patients and one control were immortalized with the E7 gene from human papilloma virus type-16 and human telomerase as previously described[28].

Immortalization of these cell lines did not affect the cellular phenotype as assessed by [^{14}C]propionate, and [^{14}C]methyltetrahydrofolate incorporation into cellular macromolecules and Cbl distributions (data not shown). The cDNA containing retroviral vector was transiently transfected into a Phoenix Amphotrophic cell line (a kind gift from G.P. Nolan, Stanford University) using an HBS/$Ca_3(PO_4)_2$ method (http://www.stanford.edu/group/nolan/retroviral_ systems/retsys.html). Patient and control fibroblasts were infected 48 h later by exposure to virus-containing medium with polybrene (4 μg/ml) and grown for 2 weeks in medium containing 100U/ml of hygromycin. Incorporation of label from radiolabeled propionate and 5-methyltetrahydrofolate and measurement of Cbl coenzyme synthesis was performed on infected cells as described above.

Modeling

The predicted amino acid sequence of MMACHC was aligned to *E. coli* TonB based on a partial sequence-based alignment of MMACHC residues 242–280 against TonB residues 119–156 of the *Salmonella typhimurium* TonB protein (P25945). The primary sequence of the C-terminal domain of *E. coli* TonB (residues 152–239) was merged with this alignment based on homology to the *S. typhimurium* sequence. The alignment of the primary sequence of MMACHC (residues 185–282) to the *E. coli* TonB sequence was used as the input for the computer program Modeller[15] to obtain a three-dimensional homology model of the C-terminal region of MMACHC. The model was refined by molecular dynamics routines, with incremental increases in simulation temperature from 150°K to 1000°K, followed by incremental temperature decreases from 1000°K to 300°K. The MMACHC model was evaluated by the program, PROCHECK[16], on an IBM Intellistation M Pro Workstation running Redhat Linux 9. The MMACHC model and the three-dimensional structure of monomeric *E. coli* TonB (PDB code 1XX3; residues 152–239) were structurally aligned in a pairwise manner by the program LSQMAN to obtain normalized rmsd statistics[17].

ACKNOWLEDGEMENTS

We dedicate this work to the memory of Jamie C. Tirone. We thank the clinicians who provided patient samples and clinical information; Nora Matiaszuk and Jocelyne Lavallée for complementation analysis; Andrei Verner and Geneviève Genaud for microsatellite genotyping; Nicole Roslin for microsatellite analysis; Maria Galvez and Junhui Liu for laboratory work; Gary Leveque, Timothy A. Johns and David Roquis for technical assistance; Sean Froese, Lisa Worgan, Kirsten Niles and Alexandre Montpetit for discussion; and Judith Kashul and David Ellis for editing. This research was supported by grants from the March of Dimes Birth Defects Foundation (6-FY01-11), Canadian Institutes of Health Research (CIHR), the Canada Foundation for Innovation to the Montreal Integrated Genomics Group for Research on Infectious Pathogens, and the Network of Centres of Excellence Program – the Canadian Genetic Diseases Network. David S. Rosenblatt is a principal investigator in the CIHR Group in Medical Genetics. This is a publication of the Hess B. and Diane Finestone Laboratory in Memory of Jacob and Jenny Finestone.

SUPPLEMENTARY NOTES

Expression

Fifty-four ESTs from multiple tissues, including testis, eye, skin, lung, uterus, ovary, mammary gland, kidney, colon and brain were listed in UniGene cluster Hs.13024. The 5' end of EST CD358853.1 is extended 361 bp upstream of the first inframe ATG initiation codon; an inframe termination codon occurs 42 bp upstream of the initiation codon in this transcript. At the 3' end, two alternate polyA tails were detected with composite mRNA species from 1465–2487 bp in size. Two splice-variant RNA species, differing in the noncoding region of exons 4 and 5, were listed (AL080062.1 and BC006122.1). A third message, AK098537.1, encodes a truncated protein missing 37 amino acids compared to AL080062.1; 131 bp of exon 4 are absent with an alternative exon 5further downstream. Thirty ESTs were listed in the mouse EST database; only four exons were annotated.

The mRNA sequence of *MMACHC* was verified by RT-PCR followed by sequencing of the cDNA transcript from the human wild-type fibroblast line MCH23. Using primers in the 5' and 3' UTRs, a fragment of 908 bp was amplified; using a sense primer extending from c.1 to c.23 with a 5'-adaptor for cloning and a 3'-UTR antisense primer, a fragment of 1070 bp was amplified.

Conservation

The human *MMACHC* gDNA sequence was used as a template for comparison with multiple species using Multi-LAGAN (Brudno, M. *et al.* LAGAN and Multi-LAGAN: efficient tools for large-scale multiple alignment of genomic DNA. *Genome Res.* **13**, 721-731 (2003)). The coding region at the nucleotide level was well conserved

from initiation codon to termination codon in mammalian species, including *Pan troglodytes*, *Canis familiaris*, *Rattus norvegicus*, and *Mus musculus*. The exon-intron splice junctions for exons 1–4 were also conserved. Nucleotide conservation in exon 5 dropped from 99% identity in *P. troglodytes* to 7% identity in *M. musculus* compared to human. gDNA sequence alignments comparing human to *Gallus gallus*, *Xenopus tropicalis*, *Tetraodon nigroviridis*, and *Caenorhabditis elegans* showed conservation, except at the 3'-terminal of exon 4. Consistent with these findings, the polypeptide sequence was well conserved in mammals but showed less sequence conservation in lower organisms at the C-terminal end.

Modeling of Mutations

The effect of two missense mutations, c.617G>C (p.R206P) and c.616C>T (p.R206W), on folding of the *MMACHC* model was examined. p.R206P aligns with P164 in *E. coli* and results in an identical residue compared to the structural template, eliminating this as a possibility for misfolding of the TonB-like domain. In order to assess the effect of introducing a tryptophan at this position, 100 homology-based models of *MMACHC*-p.R206W were generated. We did not observe any significant deterioration in Ramachandran statistics or a decrease of the overall G-factor as compared to the wild-type *MMACHC* model (data not shown). We conclude that these two missense mutations, c.617G>C (p.R206P) and c.616C>T (p.R206W), are not likely to cause misfolding of a TonB-like *MMACHC* domain, but perhaps these mutants are unable to form efficient protein-protein contacts with a cognate binding partner.

REFERENCE LIST

1. Rosenblatt, D. & Fenton, W. A. Inherited disorders of folate and cobalamin transport and metabolism in The metabolic & molecular bases of inherited disease, III, 3897-3933. McGraw-Hill, New York (2001).

2. Rosenblatt, D. S. et al. Clinical heterogeneity and prognosis in combined methylmalonic aciduria and homocystinuria (cblC). J.Inher.Met.Dis. 20, 528-538 (1997).

3. Atkinson, J. Genetic Mapping of Cobalamin C deficiency: linkage to chromosome 1p32-34. (Msc Thesis). University of Toronto, Toronto, Ontario, Canada (2003).

4. Homocysteine in Health and Disease. (eds. Carmel, R. & Jacobsen, D.W.) (Cambridge University Press, Cambridge, UK, 2001).

5. Andersson, H. C. & Shapira, E. Biochemical and clinical response to hydroxocobalamin versus cyanocobalamin treatment in patients with methylmalonic acidemia and homocystinuria (cblC). J.Pediatr. 132, 121-124 (1998).

6. Mellman, I., Willard, H. F., Youngdahl-Turner, P., & Rosenberg, L. E. Cobalamin coenzyme synthesis in normal and mutant fibroblasts: Evidence for a processing enzyme activity deficient in cblC cells. J. Biol.Chem. 254, 11847-11853 (1979).

7. Templeton, A. R. et al. Recombinational and mutational hotspots within the human lipoprotein lipase gene. Am .J.Hum.Genet. 66, 69-83 (2000).

8. Stephens, M., Smith, N. J., & Donnelly, P. A new statistical method for haplotype reconstruction from population data. Am.J.Hum.Genet 68, 978-989 (2001).

9. Stephens, M. & Donnelly, P. A comparison of Bayesian methods for haplotype reconstruction. Am.J.Hum.Genet 73, 1162-1169 (2003).

10. Maquat, L. E. Nonsense-mediated mRNA decay:splicing, translation and mRNP dynamics. Nat.Rev.Mol.Cell Biol. 5, 89-99 (2004).

11. Drennan, C. L., Huang, S., Drummond, J. T., Matthews, R. G., & Ludwig, M. L. How a protein binds B_{12}: a 3.0 A X-ray structure of B_{12} -binding domains of methionine synthase. Science 266, 1669-1674 (1994).

12. Chang, C., Mooser, A., Plückthun, A., & Wlodawer, A. Crystal structure of the dimeric C-terminal domain of TonB reveals a novel fold. J. Biol.Chem. 276, 27535-27540 (2001).

13. Ködding, J. et al. Crystal structure of a 92-residue C-terminal fragment of TonB from Escherichia coli reveals significant conformational changes compared to structures of smaller TonB fragments. J.Biol.Chem. 280, 3028 (2005).

14. Peacock, R. S., Weljie, A. M., Howard, S. P., Price, F. D., & Vogel, H. J. The solution structure of the C-terminal domain of TonB and interaction studies with TonB box peptides. J.Mol.Biol. 345, 1185-1197 (2005).

15. Sali, A. & Blundell, T. L. Comparative protein modelling by satisfaction of spatial restraints. J.Mol.Biol. 234, 779-815 (1993).

16. Laskowski, R. A., MacArthur, M. W., & Thornton, J. M. PROCHECK: a program to check the stereochemical quality of protein structure. J.Appl.Cryst. 26, 283-291 (1993).

17. Kleywegt, G. J. Experimental assessment of differences between related protein crystal structures. Acta.Cryst.D.Biol.Crystallogr. 55, 1878-1884 (1999).

18. Bell, P. E., Nau, C. D., Brown, J. T., Konisky, J., & Kadner, R. J. Genetic suppression demonstrates interaction of TonB protein with outer membrane transport proteins in Escherichia coli. J.Bacteriol. 172, 3826-3829 (1990).

19. Cadieux, N. & Kadner, R. J. Site-directed disulfide bonding reveals an interaction site between energy-coupling protein TonB and BtuB, the outer membrane cobalamin transporter. Proc.Natl.Acad.Sci.U.S.A. 96, 10673-10678 (1999).

20. Rosenblatt, D. S., Hosack, A., Matiaszuk, N. V., Cooper, B. A., & Laframboise, R. Defect in vitamin B12 release from lysosomes: newly described inborn error of vitamin B12 metabolism. Science 228(4705), 1319-1321 (1985).

21. Pezacka, E. H. & Rosenblatt, D. S. Intracellular metabolism of cobalamin. Altered activities of b-axial-ligand transferase and microsomal cob(III)alamin reductase in cblC and cblD fibroblasts. in Advances in Thomas Addison's Diseases. 315-323. Journal of Endocrinology, Bristol, London (1994).

22. Brudno, M. et al. LAGAN and Multi-LAGAN: efficient tools for large-scle multiple alignment of genomic DNA. Genome Res. 13, 721-731 (2003).

23. Watkins, D. Cobalamin metabolism in methionine-dependent human tumour and leukemia cell lines. Clin.Invest.Med. 21, 151-158 (1998).

24. Watkins, D., Matiaszuk, N., & Rosenblatt, D. S. Complementation studies in the cblA class of inborn error of cobalamin metabolism: evidence for interallelic

complementation and for a new complementation class (cblH). J.Med.Genet. 37, 510-513 (2000).

25. Ewing, B., Hillier, L., Wendl, M. C., & Green, P. Base-calling of automated sequencer traces using Phred I. Accuracy assesment. Genome Res. 8, 175-185 (1998).

26. Gordon, D., Abajian, C., & Green, P. Consed: A graphical tool for sequence finishing. Genome Res. 8, 195-202 (1998).

27. Miller, A. D. & Buttimore, C. Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. Mol.Cell.Biol. 6, 2895-2902 (1986).

28. Yao, J. & Shoubridge, E. A. Expression and functional analysis of SURF1 in Leigh syndrome patients with cytochrome C oxidase deficiency. Hum.Mol.Genet 8, 2541-2549 (1999).

29. Horton, P. & Nakai, K. Better prediction of protein cellular localization sites with the k nearest neighbors classifier. Proc. Int. Conf. Intell. Syst. Mol. Biol. 5, 147-152 (1997).

30. Kong, X. & Matise, T. C. MAP-O-MAT: intenet-based linkage mapping. Bioinformatics 21, 557-559 (2005).

FIGURE LEGENDS

Figure 1. (a) Chromosomal Location. The 6.6 Mb interval on chromosome 1 defined by Atkinson[3]. The physical position of markers used for homozygosity and haplotype analysis are shown. The position of markers is according to NCBI build 35.1. The genomic location of MMACHC is specified in chromosome 1 genomic contig NT_004511.17. The gene maps to chromosome region 1p34.1 and is transcribed in the direction from telomere to centromere. Sequence-tagged site entries include RH69651, SHGC-146777, RH36426, and SHGC-33398.

(b) Gene Structure. Consisting of five exons, MMACHC includes 10,736 bp of genomic DNA, as defined by reference sequence NM_015506.1. Exons 1–4 are predicted to encode an 849 bp message from initiation to termination codons; the 3' end of exon 4 and exon 5 are untranslated. All exon-intron boundaries obey the AG-GT rule and preserve the open reading frame (ORF) as predicted by the cDNA sequence. The coding regions are indicated as solid boxes; exon sizes are indicated in bp below the gene structure, and intron sizes in bp are indicated between exons. All coding exons are inframe. The cDNA has an 846 bp ORF encoding a polypeptide of 282 amino acids with a predicted molecular weight of 31.7 kDa. The program PSORT II[29] predicts that the protein localizes to the cytoplasm. NCBI reference ID numbers for SNPs and DIPs identified during mutation analysis are shown; rsJLE02 is a DIP in exon 5 (c.*855_856delCT). MMACHC is head to head with an uncharacterized gene directly upstream supported by EST CR606750.1. Further upstream is a testis-specific protein kinase gene (TESK2: NM_007170.1). Tail to tail with MMACHC is a gene that encodes a member of the peroxiredoxin family (PRDX1: NM_181697.1). Synteny can be observed between human, mouse and rat DNA sequences surrounding this region. The human-mouse-rat homology map links human chromosome region 1p34.1 to chromosomes 4 and 5 of mouse and rat, respectively (http://www.ncbi.nlm.nih.gov/Homology/).

(c) MMACHC Mutations. The location of putative disease-causing protein-truncating mutations are shown above the composite cDNA; nontruncating mutations are shown below.

Figure 2. Transduction of MMACHC. Two cblC patient fibroblast cell lines (from a patient homozygous for c.271dupA (line 1); and a patient compound heterozygous for c.271dupA and c.331C>T (line 2)) and a control (MCH64) fibroblast cell line were immortalized and infected with wild-type MMACHC.

(a) [^{14}C]methyltetrahydrofolate incorporation into cellular macromolecules in infected and uninfected cblC and control cell lines. Values are means ± SD of three replicates.

(b) [^{14}C]propionate incorporation into cellular macromolecules in infected and uninfected cblC and control cell lines. Values are means ± SD of three replicates.

(c) Cellular Cbl distributions in infected and uninfected cblC and control cell lines. OHCbl: hydroxylcobalamin or aqualcobalamin; CNCbl: Cyanocobalamin; AdoCbl: Adenosylcobalamin; MeCbl: Methylcobalamin. Total uptake of Cbl (pg/10^6 cells) was measured. Control: 4.4; Control + MMACHC: 4.9; Line 1: 4.8; Line 1 + MMACHC: 16.5; Line 2: 4.8; Line 2 + MMACHC: 9.6.

Figure 3. Haplotypes inferred from 54 CEPH individuals and from 99 cblC patients homozygous for MMACHC mutations. (a) The frequencies and standard errors (SE) estimated for the four most common haplotypes among 19 haplotypes estimated in a sample of 54 CEPH individuals. Bold font indicates alleles of eight common polymorphisms; italic font indicates alleles of five rare variants found only in cblC patients. The nucleotide position of each variant is numbered relative to the A of the ATG initiation codon; "-" indicates 5' of the initiation codon and "*" indicates 3' of the termination codon. The variants corresponding to the column headings are: c.1-302T>G (rs3748643); c.1-234A>G (rs3748644); c.1-50A>G; c.181C>T (p.R61W); c.321G>A (p.V107 silent substitution) (rs2275276); c.800G>A (p.R267Q); c.*126dupT; c.*279A>G (rs9729395); c.*855_856delCT (rsJLE02); c.*1362G>A (rs12728919); c.*1376_1377insT (rs5773883); c.*1585G>C (rs7903); and c.*1700G>A (rs1044717), respectively.

(b) Haplotypes of 99 cblC patients homozygous for MMACHC mutations. N is the number of alleles of a given haplotype. Alleles outlined in boxes indicate differences between alleles carrying the same MMACHC mutation; shaded boxes indicate rare alleles found only in the cblC patients. ND = no data.

Figure 4. (a) Polypeptide sequence alignment of MMACHC and its orthologs. ClustalW and Genedoc programs were used to align and shade. Percent identities, in parentheses below, were compared to the Homo Sapiens sequence NP_056321.1. Codon 100 was verified experimentally and differs from the reference sequence NM_015506.1 or NP_056321.1. The nucleotide sequence GGA (glycine) was determined to be GAA (glutamic acid) in all our sequences. Aligned sequences include Pan troglodytes XP_524565.1 (99%); Canis familiaris XP_539631.1 (87%); Bos taurus NP_001015588.1 (87%), Rattus norvegicus XP_233418.2 (82%), Mus musculus BAB28451.1 (82%), Gallus gallus CAG31572.1 (51%), Xenopus tropicalis CR448475.1 (49%), Tetraodon nigroviridis CAG01682.1 (41%), and Caenorhabditis elegans AAP68926.1 (26%). Amino acids conserved in all species are shaded in black. Gray shading indicates 80-90% conservation. The putative vitamin B_{12}-binding site is shown including sequence with identity to a portion of the classical B_{12}-binding site (^{122}HxxG-X_{29}-GG). The predicted effect of identified mutations on the protein product and the location of the MMACHC TonB domain are indicated.

(b) Ribbon representation of the homology model of the C-terminus of MMACHC to TonB. Strands are represented as flat arrows, helices as flat coils, and loops as thin tubes. The color is ramped according to the relative position between the N-terminus (blue) and C-terminus (red). The model of the MMACHC C-terminal domain has a noncontiguous four-stranded β-sheet with a 12-residue α-helix located between the exit of β-strand 2 and the entrance to β-strand 3. This helix lies against the plane of the central β-sheet.

(c) Cα superposition of the MMACHC homology model (red) and the three-dimensional NMR structure of the monomeric C-terminal domain of TonB (PDB code: 1XX3) (blue). The arrow indicates the loop between residues 198–203.

Figure 1

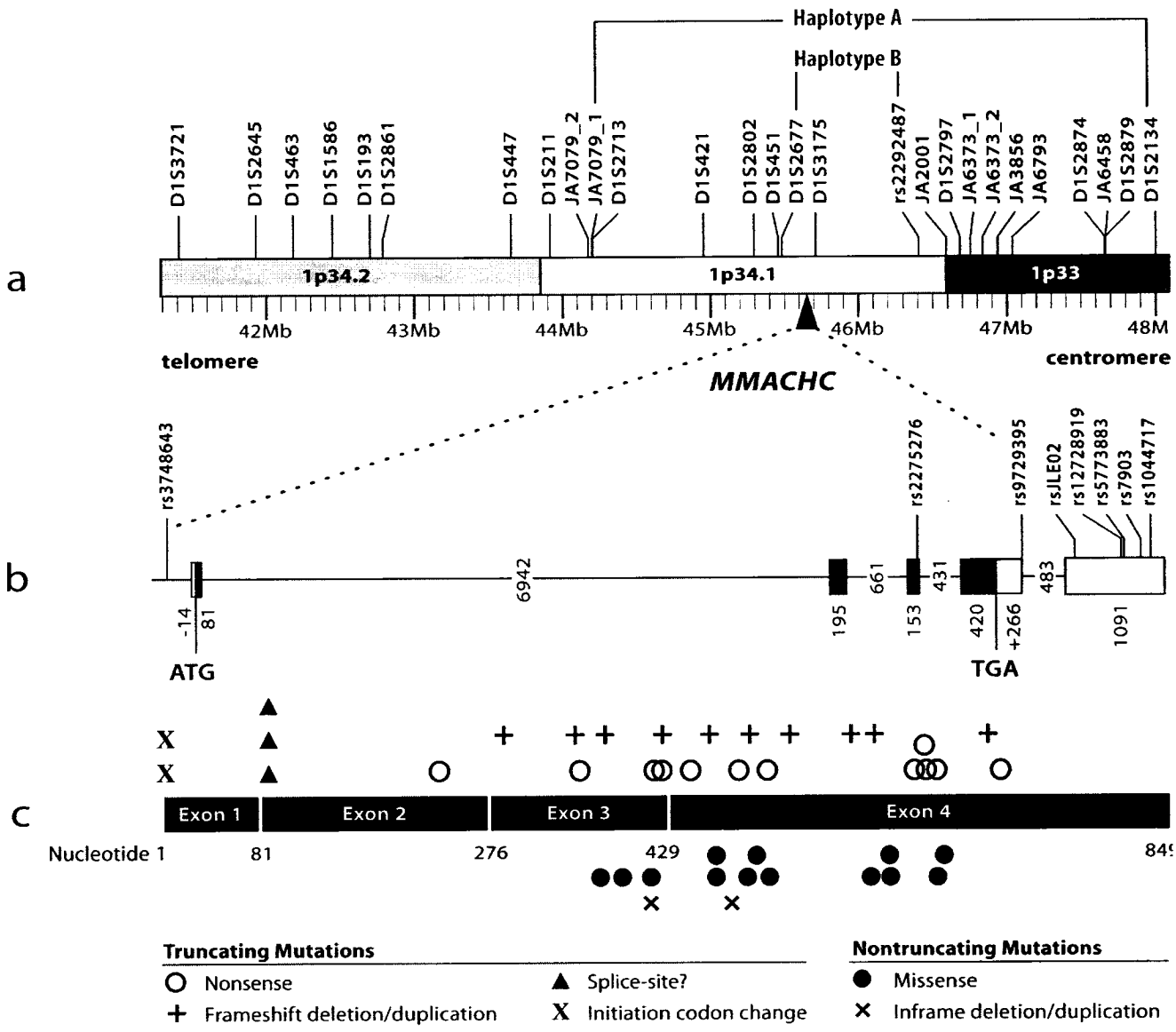
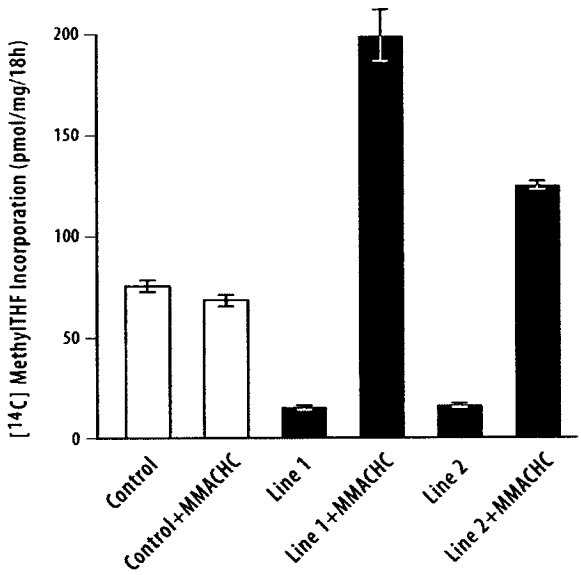
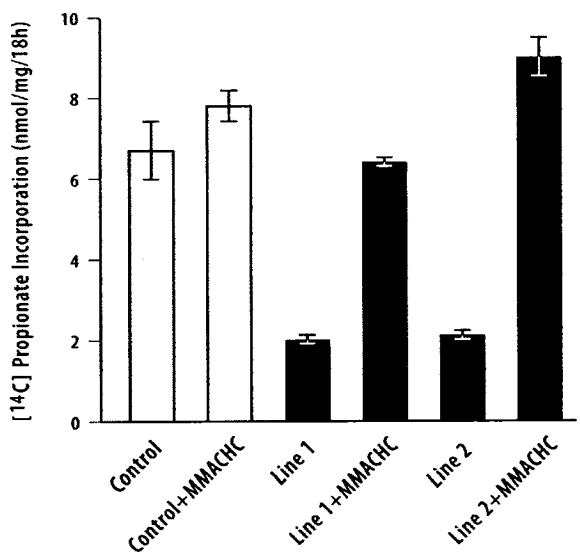

Haplotype A
Haplotype B
D1S3721
D1S2645
D1S463
D1S1586
D1S193
D1S2861
D1S447
D1S211
JA7079_2
JA7079_1
D1S2713
D1S421
D1S2802
D1S451
D1S2677
D1S3175
rs2292487
JA2001
D1S2797
JA6373_1
JA6373_2
JA3856
JA6793
D1S2874
JA6458
D1S2879
D1S2134
a
1p34.2
1p34.1
1p33
42Mb
43Mb
44Mb
45Mb
46Mb
47Mb
48M
telomere
centromere
MMACHC
rs3748643
rs2275276
rs9729395
rsJLE02
rs12728919
rs5773883
rs7903
rs1044717
b
6942
661
431
483
195
153
420
+266
1091
-14
81
ATG
TGA
c
Exon 1
Exon 2
Exon 3
Exon 4
Nucleotide 1
81
276
429
849
Truncating Mutations
Nonsense
Frameshift deletion/duplication
Splice-site?
Initiation codon change
Nontruncating Mutations
Missense
Inframe deletion/duplication

Figure 2

a


[14C] MethylTHF Incorporation (pmol/mg/18h)
200
150
100
50
0
Control
Control+MMACHC
Line 1
Line 1+MMACHC
Line 2
Line 2+MMACHC

b


[14C] Propionate Incorporation (nmol/mg/18h)
10
8
6
4
2
0
Control
Control+MMACHC
Line 1
Line 1+MMACHC
Line 2
Line 2+MMACHC

c

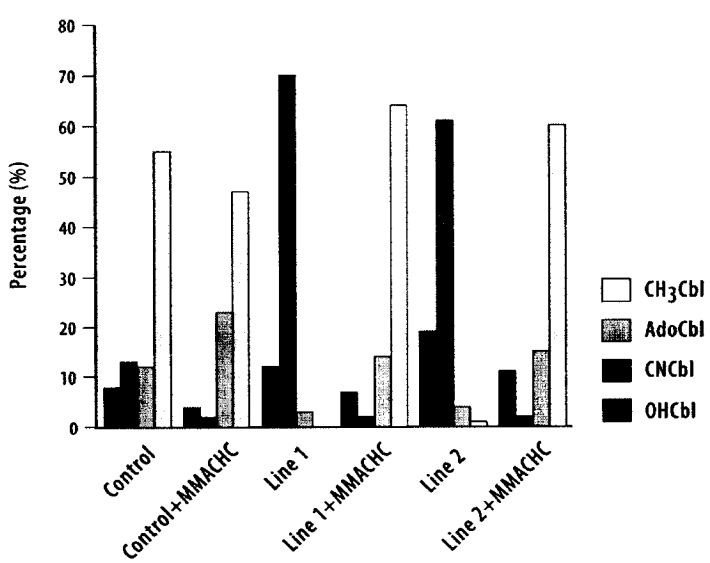

Percentage (%)
80
70
60
50
40
30
20
10
0
Control
Control+MMACHC
Line 1
Line 1+MMACHC
Line 2
Line 2+MMACHC
CH3Cbl
AdoCbl
CNCbl
OHCbl

Figure 3

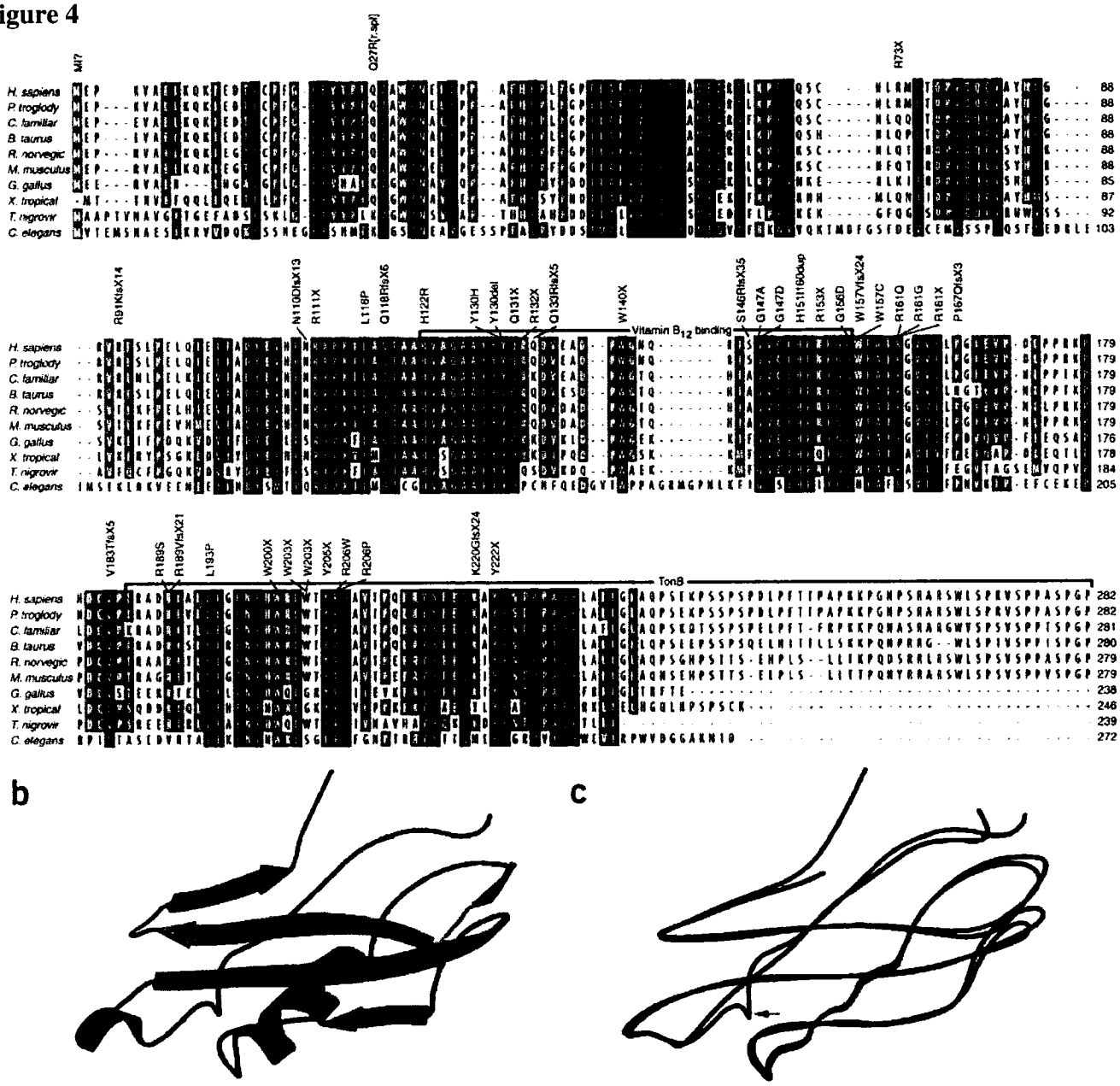
a Haplotypes based on 54 CEPH individuals

	Nucleotide position of variant														
	-302	-234	-50	181	321	800	*126	*279	*855	*1362	*1376	*1585	*1700	Freq	SE
Haplotype #1	T	A	A	C	A	G	T	A	ΔCT	G	T	C	G	0.29	0.010
Haplotype #2	T	A	A	C	G	G	T	G	ΔCT	G	T	G	G	0.27	0.004
Haplotype #3	G	A	A	C	G	G	T	A	CT	G	–	G	A	0.25	0.007
Haplotype #4	T	A	A	C	A	G	T	A	ΔCT	A	T	C	G	0.11	0.009
Fifteen other haplotypes combined														0.08	

b Haplotypes of 99 patients who were homozygous for an *MMACHC* mutation.

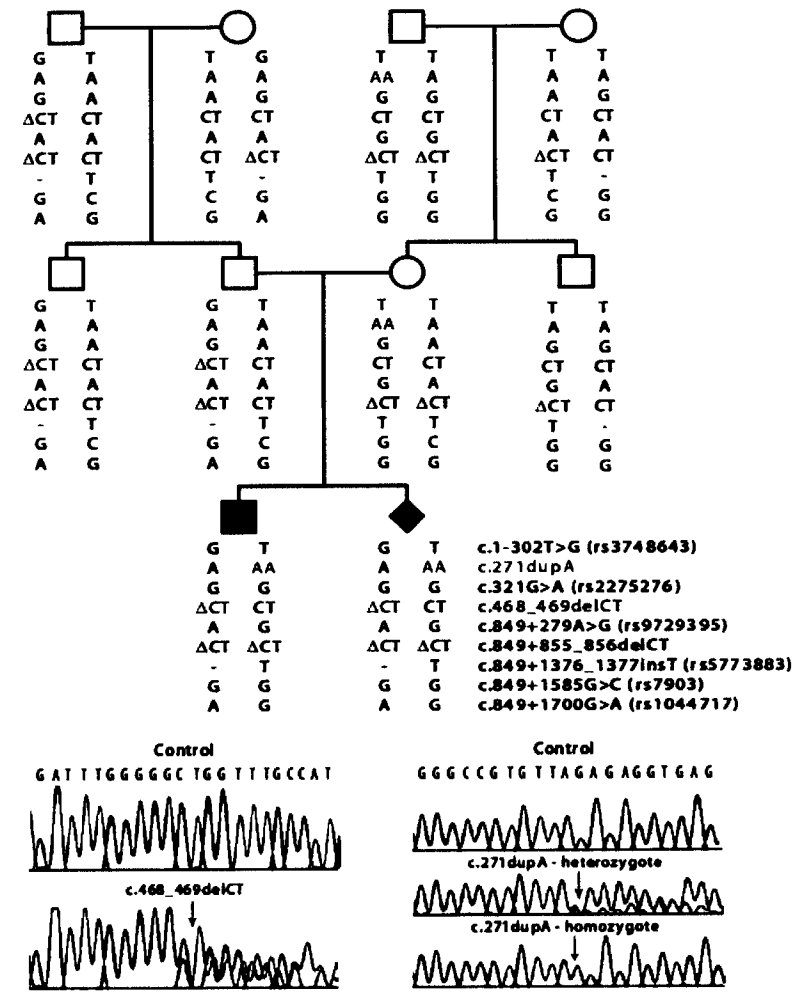
Mutation	Haplotype	-302	-234	-50	181	321	800	*126	*279	*855	*1362	*1376	*1585	*1700	N
c.3G>A	#2	T	A	A	C	G	G	T	G	ΔCT	G	T	G	G	6
c.3G>A	#3	G	A	A	C	G	G	T	A	CT	G	–	G	A	2
c.217C>T	#1	T	A	A	C	A	G	T	A	ΔCT	G	T	C	G	4
c.217C>T	#3	G	A	A	C	G	G	T	A	CT	G	–	G	A	2
c.271dupA	#2	T	A	A	C	G	G	T	G	ΔCT	G	T	G	G	94
c.271dupA		G	A	A	C	G	G	T	G	ΔCT	G	T	G	G	4
c.271dupA		T	A	A	C	G	A	T	G	ΔCT	G	T	G	G	3
c.271dupA		T	A	G	C	G	G	T	G	ΔCT	G	T	G	G	1
c.331C>T	#1	T	A	A	C	A	G	T	A	ΔCT	G	T	C	G	10
c.331C>T		T	A	A	T	G	G	T	A	ΔCT	G	T	C	G	4
c.394C>T	#1	T	A	A	C	A	G	T	A	ΔCT	G	T	C	G	10
c.394C>T		T	A	A	C	G	G	T	A	ΔCT	G	T	C	G	12
c.80A>G	#1	T	A	A	C	A	G	T	A	ΔCT	G	T	C	G	2
c.328_331delAACC	#1	T	A	A	C	A	G	T	A	ΔCT	G	T	C	G	2
c.435_436delAT	#2	T	A	A	C	G	G	T	G	ΔCT	G	T	G	G	2
c.440G>C		T	A	A	C	G	G	T	A	ΔCT	G	T	C	G	2
c.440G>A		T	A	A	C	G	G	TT	G	ΔCT	G	T	G	G	6
c.547_548delGT	#2	T	A	A	C	G	G	T	G	ΔCT	G	T	G	G	8
c.578T>C	#1	T	A	A	C	A	G	T	A	ΔCT	G	T	C	G	2
c.608G>A	#2	T	A	A	C	G	G	T	G	ΔCT	G	T	G	G	6
c.609G>A		G	G	A	C	G	G	T	A	CT	G	–	G	A	10
c.615C>G	#3	G	A	A	C	G	G	T	A	CT	G	–	G	A	2
c.616C>T		T	A	A	C	ND	G	T	A	ΔCT	G	T	C	G	2
c.617G>C	#2	T	A	A	C	G	G	T	G	ΔCT	G	T	G	G	2

Figure 4


MI?
Q27R[r.spl]
R73X
R91KfsX14
N110DfsX13
R111X
L118P
Q118RfsX6
H122R
Y130H
Y130delI
Q131X
R132X
Q133RfsX5
W140X
Vitamin B12 binding
S146RfsX35
G147A
G147D
H151fs60dup
R153X
Q156D
W157VfsX24
W157C
R161Q
R161G
R161X
P167QfsX3
V183TfsX5
R189S
R189VfsX21
L193P
W200X
W203X
W203X
Y205X
R206W
R206P
K220GfsX24
Y222X
TonB
H. sapiens
P. troglody
C. familiar
B. taurus
R. norvegic
M. musculus
G. gallus
X. tropical
T. nigrovir
C. elegans

b
c

Supplementary Figure 1. The pedigree of a family segregating *MMACHC* mutations.



Supplementary Figure 1. One affected child and one affected fetus were diagnosed with *cblC*. Genotype data for informative SNPs are shown. Sequence chromatograms are shown below the pedigree.

TABLES

Table 1. MMACHC mutations identified in 204 cblC patients

Forty-two different mutations were identified in the MMACHC gene. The predicted effect on protein or mRNA is shown. Mutations are listed in the order in which they occur within the gene. DNA mutation numbering is based on cDNA sequence: +1 corresponds to the A of the ATG translation initiation codon. The number of disease alleles identified for each mutation is listed (NM_015506.1).

Mutation	Predicted Effect on Protein or mRNA	No. of alleles detected in 204 cblC patients
c.1A>T	p.M1?	1
c.3G>A	p.M1?	10
c.80A>G	p.Q27R[r.spl?]	2
c.81+1G>A	r.spl?	1
c.82-9_12delTTTC	r.spl?	2
c.217C>T	p.R73X	9
c.271dupA	p.R91KfsX14	165
c.328_331delAACC	p.N110DfsX13	8
c.331C>T	p.R111X	36
c.347T>C	p.L116P	2
c.352delC	p.Q118RfsX6	4
c.365A>G	p.H122R	1
c.388T>C	p.Y130H	2
c.388_390delTAC	p.Y130del	6
c.391C>T	p.Q131X	1
c.394C>T	p.R132X	34
c.398_399delAA	p.Q133RfsX5	1
c.420G>A	p.W140X	2
c.435_436delAT	p.I145IfsX36	2
c.440G>A	p.G147D	9
c.440G>C	p.G147A	3
c.450_479dup30	p.I150_A159dup	1
c.457C>T	p.R153X	4
c.467G>A	p.G156D	1
c.468_469delCT	p.G156GfsX25	2
c.471G>C	p.W157C	1
c.481C>T	p.R161X	4
c.481C>G	p.R161G	1
c.482G>A	p.R161Q	8
c.500delC	p.P167QfsX3	1
c.547_548delGT	p.V183TfsX5	8
c.565C>A	p.R189S	1
c.565delC	p.R189VfsX21	1
c.578T>C	p.L193P	3
c.600G>A	p.W200X	1
c.608G>A	p.W203X	6
c.609G>A	p.W203X	12
c.615C>G	p.Y205X	11
c.616C>T	p.R206W	2
c.617G>C	p.R206P	2
c.658_659delAA	p.K220GfsX24	1
c.666C>A	p.Y222X	3
Not identified	–	33

Table 2. Age of diagnosis of patients with different combinations of MMACHC mutations

Patient Genotype			Age of Diagnosis				
Mutation1	Mutation2	No. of Patients	<1 year	1-4 years	4-10years	10-20 Years	>20 years
c.1A>T	c.331C>T	1	1				
c.3G>A	c.3G>A	4	2		1	1	
c.3G>A	c.331C>T	1		1			
c.80A>G	c.80A>G	1	1				
c.81+1G>A	c.482G>A	1				1	
c.82-9_12delTTTC	c.271dupA	2			1	1	
c.217C>T	c.217C>T	3	3				
c.217C>T	c.271dupA	2	2				
c.217C>T	c.609G>A	1			1		
c.271dupA	c.271dupA	46	44	2			
c.271dupA	c.331C>T	12	12				
c.271dupA	c.347T>C	2					2
c.271dupA	c.352delC	3	3				
c.271dupA	c.388_390delTAC	2	2				
c.271dupA	c.391C>T	1	1				
c.271dupA	c.394C>T	4	1		3		
c.271dupA	c.420G>A	1	1				
c.271dupA	c.440G>A	3	3				
c.271dupA	c.440G>C	1					1
c.271dupA	c.457C>T	2	2				
c.271dupA	c.481C>T	2	2				
c.271dupA	c.482G>A	3				2	1
c.271dupA	c.565C>A	1			1		
c.271dupA	c.600G>A	1	1				
c.271dupA	c.609G>A	1	1				
c.271dupA	c.615C>G	5	5				
c.271dupA	c.658_659delAA	1	1				
c.271dupA	c.666C>A	1	1				
c.328_331delAACC	c.328_331delAACC	2	1	1			
c.328_331delAACC	c.331C>T	1	1				
c.328_331delAACC	c.394C>T	2	1	1			
c.328_331delAACC	c.467G>A	1	1				
c.328_331delAACC	c.482G>A	1		1			
c.331C>T	c.331C>T	9	9				
c.331C>T	c.388_390delTAC	1	1				
c.331C>T	c.420G>A	1	1				
c.331C>T	c.615C>G	2	2				
c.331C>T	c.666C>A	1	1				
c.388T>C	c.471G>C	1		1			
c.394C>T	c.328_331delAACC	2	1	1			
c.394C>T	c.394C>T	11	1	1	3	6	
c.394C>T	c.398_399delAA	1	1				
c.394C>T	c.450_479dup30	1	1				
c.394C>T	c.457C>T	1	1				
c.394C>T	c.468_469delCT	1	1				
c.394C>T	c.666C>A	1				1	
c.435_436delAT	c.435_436delAT	1	1				
c.440G>A	c.440G>A	3	3				
c.440G>C	c.440G>C	1		1			
c.481C>G	c.481C>T	1	1				
c.481C>T	c.565delC	1	1				
c.482G>A	c.615C>G	1			1		
c.547_548delGT	c.547_548delGT	4	4				
c.578T>C	c.578T>C	1	1				
c.578T>C	c.615C>G	1	1				
c.608G>A	c.608G>A	3	3				
c.609G>A	c.609G>A	5	3	1		1	
c.615C>G	c.615C>G	1	1				
c.617G>C	c.617G>C	1				1	

Patients with no clinical data or samples ascertained by newborn screening or prenatal diagnosis were not included.

Supplementary Table 1. Markers used for homozygosity mapping and haplotype analyses

No.	Marker	Physical Position	Marshfield (cM)	DeCODE (cM)	Rutgers (cM)	Hetetrozygosity
1*	D1S3721	41395560	72.59	65.87	76.12	0.90 (989)
2	D1S2645	41925657	73.21	ND	76.72	0.78 (95)
3*	D1S463	42181826	73.21	66.44	76.72	0.78 (850)
4	D1S1586	42446260	73.21	66.44	76.72	0.69 (692)
5	D1S193	42688369	73.21	66.64	76.9	0.83 (784)
6	D1S2861	42775945	72.59	66.64	76.9	0.72 (781)
7	D1S447	43626627	73.81	ND	78.1	0.77 (131)
8*	D1S211	43900434	73.81	ND	78.1	0.85 (131)
9*	JA7079_2	44153337	ND	ND	ND	ND
10*	JA7079_1	44165893	ND	ND	ND	ND
11	D1S2713	44182569	73.81	67.87	78.1	0.77 (917)
12	D1S421	44925737	73.81	68.9	79.35	0.67 (600)
13	D1S2802	45265050	75.66	ND	79.35	0.69 (108)
14	D1S451	45426354	75.66	68.9	79.35	0.78 (620)
15	D1S2677	45461176	75.66	ND	79.35	0.76 (146)
16	D1S3175	45686718	76.27	68.9	79.35	0.52 (515)
17*	JA2001	46554293	ND	ND	ND	ND
18	D1S2797	46645460	75.66	68.9	79.35	0.78 (792)
19*	JA6373_1	46717992	ND	ND	ND	ND
20*	JA6373_2	46801196	ND	ND	ND	ND
21*	JA3856	46898146	ND	ND	ND	ND
22*	JA6793	46994166	ND	ND	ND	ND
23	D1S2874	47620140	75.66	70.35	80.58	0.78 (580)
24*	JA6458	47626311	ND	ND	ND	ND
25	D1S2879	47626326	75.66	70.35	80.58	0.71 (666)
26*	D1S2134	47993241	75.66	71.29	81.37	0.82 (801)

Markers used for homozygosity and haplotype analyses are listed in order based on the physical position according to NCBI build 35.1. JA markers were identified by Janet Atkinson[3]. Marker numbers labeled with an asterix were used to construct haplotype A (Fig. 1a). Heterozygosity values are from MAP-O-MAT (Kong, X. & Matise, T.C. MAP-O-MAT: internet-based linkage mapping. *Bioinformatics* **21**, 557-559 (2005)) and were calculated based on the CEPH families plus Icelandic families from deCODE Genetics. Where available the number in parentheses refers to the total number of informative meioses. ND: not determined.

Supplementary Table 2. Primers used to amplify the *MMACHC* gene from gDNA and cDNA

Exon	5'-3' Sense	5'-3' Antisense	PCR Product Size (bp)
1	GGGATACCGTGATGATACGC	GAACCCAGGAGGATCAGAGG	680
2	TGCATCACATAGCGTCAGTG	AGCCTGGCTTTAGGGTATCA	467
3	TCATGTTTTCCCTTCTGAGGA	CAAAGCTAATTTGTTCTGGGTTG	395
4a	AGGCCTAGCTTGCAATGATG	GAAGGCAGATGGGAATTCTG	694
4b	TTTGGCAAAGCAAAAGGTT	CAAGATGGGTGGATCACGA	395
5a	AGCCTGGCCAATACAGTGAA	AGCCTTCCCTTGGTTCTAGC	683
5b	ACCATTTTGGGAGGCTGAG	GGGCAGGCTACTGGTTTGTA	685
cDNA	CAGCAAGCTCAGCGTGTAAC	CCACCATAAATCAGGGTCCA	908
[a]Clone	caccATGGAGCCGAAAGTCGCAGAGCT	GAAGGCAGATGGGAATTCTG	1047
[b]Probe	AGGTTTACCCCTTCCAGGTG	AGGCCTTCTGCTCTTCTGAG	600

Exons 4 and 5 were each amplified in two overlapping segments designated "a" and "b".
[a] Clone primers were used to amplify a PCR product for cloning. The sense primer contains a 5'adaptor (cacc) for incorporation into vector.
[b] Probe primers were used to amplify a probe for Northern blot analysis.

CHAPTER 5.0: Combined methylmalonic aciduria and homocystinuria (*cblC*): Phenotype-genotype correlations and ethnic-specific observations

Chantal F. Morel, M.D., Jordan P. Lerner- Ellis, and David S. Rosenblatt, M.D.

Department of Human Genetics and Division of Medical Genetics, Department of Medicine, McGill University, Montreal, Quebec, Canada (C.F.M, J.P. L-E. and D.S.R.)

Published: Mol Genet Metab. 2006 Aug;88(4):315-21. Epub 2006 May 22. PMID: 16714133

Since the original description of *cblC* disease in 1969 (48), at least 17 articles have been written describing the presentation, clinical and biochemical phenotypes. With the identification of the *MMACHC* gene, it was now possible to review these cases in the literature and describe the underlying causal mutations in these patients. The following manuscript reviews these previous cases ascribing mutations to them and linking phenotype to genotypes in 37 individuals.

ABSTRACT:

Methylmalonic aciduria and homocystinuria, *cblC* type (MIM 277400), is the most frequent inborn error of vitamin B_{12} (cobalamin, Cbl) metabolism, caused by an inability of the cell to convert Cbl to both of its active forms (MeCbl, AdoCbl). Although considered a disease of infancy, some patients develop symptoms in childhood, adolescence or adulthood. The gene responsible for *cblC*, *MMACHC*, was recently identified. We studied phenotype-genotype correlations in thirty-seven patients from published case-reports, representing most of the landmark descriptions of this disease. 25/37 had early-onset disease, presenting in the first 6 months of life: 17/25 were found to be either homozygous for the c.271dupA mutation (n=9) or for the c.331C>T mutation (n=3), or compound heterozygotes for these 2 mutations (n=5). 9/12 late-onset cases presented with acute neurological symptoms: 4/9 were homozygous for the c.394C>T mutation, 2/9 were compound heterozygotes for the c.271dupA and c.394C>T mutations, and 3/9, for the c.271dupA mutation and a missense mutation. Several observations on ethnic origins were noted: the c.331C>T mutation is seen in Cajun and French-Canadian patients and the c.394C>T mutation is common in the Asiatic-Indian/Pakistani/Middle Eastern populations. The recognition of phenotype-genotype correlations and the association of mutations with specific ethnicities will be useful for identification of disease-causing mutations in *cblC* patients, for carrier detection and prenatal diagnosis in families where mutations are known, and in setting up initial screening programs in molecular diagnostic laboratories. Further study into disease mechanism of specific mutations will help to understand phenotypic presentations and the overall pathogenesis in *cblC* patients.

KEYWORDS: *clbC*, methylmalonic aciduria, homocystinuria, *MMACHC*, genotype, phenotype.

In 1969, McCully proposed that hyperhomocysteinemia plays a role in the pathogenesis of arteriosclerosis [1]. This hypothesis was elaborated based on the pathological evidence of similar arterial damage seen in two patients with different inborn errors of metabolism both presenting with hyperhomocysteinemia: one "classic" homocystinuria, (cystathionine synthase deficiency, MIM 236200) and a second with a previously undescribed abnormality of cobalamin metabolism resulting in homocystinuria, cystathioninuria and methylmalonic aciduria. The second patient was in fact the very first cblC patient described in the literature [2, 3]. cblC (MIM 277400) is the most common inborn error of cobalamin metabolism, with over 300 patients diagnosed.

Vitamin B_{12} (cobalamin, Cbl), in the form of the cofactors methylcobalamin (MeCbl) and adenosylcobalamin (AdoCbl), is required by the cytoplasmic enzyme methionine synthase and by the mitochondrial enzyme methylmalonyl-CoA mutase, respectively. Inherited disorders of intracellular cobalamin metabolism are rare conditions, and have been classified on the basis of somatic cell complementation as belonging to eight different mutant groups (cblA to cblH) [4]. Functional deficiencies of methionine synthase lead to homocystinuria and elevated total plasma homocysteine (cblE, cblG and cblD variant 1), while a primary defect in mutase or in the synthesis of AdoCbl lead to methylmalonic aciduria (cblA, cblB, cblD variant 2 and cblH). A deficiency of the enzymes involved in the early steps of cellular vitamin B_{12} metabolism lead to accumulation of both homocysteine and methylmalonic acid in blood and urine (cblC, cblD and cblF). Figure 1 summarizes these conditions.

cblC was originally thought to be primarily a disease of infancy, presenting with failure to thrive, acute neurological deterioration, mental retardation, retinopathy, multisystem organ dysfunction and haematological abnormalities including megaloblastic anemia [4]. In 1984, Shinnar and Singer [5] reported a previously asymptomatic 14 year old girl diagnosed with cblC who presented with an acute onset of neurological symptoms, including dementia, myelopathy and motor neuron disease: this case-report was pivotal in establishing the heterogeneity of this condition. In 1997, Rosenblatt et al reviewed 50 cblC patients, of whom 6 patients had late-onset [6]. The authors concluded that the late-onset patients had better survival and response to treatment, and less

neurological sequelae compared to the early-onset patients. Various case reports describing late-onset cases followed suite [7, 8, 9, 10, 11, 12, 13, 14].

Recently, we identified the gene responsible for this condition by homozygosity mapping and haplotype analysis [15]. The gene was named MMACHC (for methylmalonic aciduria cblC type with homocystinuria): it is comprised of 4 coding exons and a 5th non-coding exon. Molecular modeling predicts that the C-terminal region of the gene product folds similarly to TonB, a bacterial protein involved in energy transduction for vitamin B_{12}-uptake.

SUBJECTS AND METHODS

Subjects:

For the past 22 years cultured fibroblasts from patients suspected of having an inborn error of Cbl metabolism have been sent to our laboratory for clinical diagnosis and characterization. A unique identification code was assigned to cell lines by the Repository for Mutant Human Cell Strains at the Montreal Children's Hospital (http://www.cellbank.mcgill.ca). Referring physicians indicated whether cell lines should not be used to pursue research into the origin of these diseases. The procedure was approved by the Royal Victoria Hospital Research Ethics Board. Thirty-seven patients in this collection have been the subjects of previous publications [16, 5, 7, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 8, 28, 9, 10, 11, 29, 12, 30, 31, 13, 32, 14]. The diagnosis of cblC was made based on Cbl distribution studies as per the methods outlined in Rosenblatt et al [33], as well as incorporation of label from [^{14}C]propionate and 5-[^{14}C]methyltetrahydrofolate into cellular macromolecules and complementation studies as outlined by Watkins et al [34]. This paper focuses on published case-reports of cblC patients, assigns causal mutations to many of the reported individuals and examines phenotypic correlations.

Mutation analysis:

The open-reading frame and flanking exon sequences of the MMACHC gene, and the non-coding portion of exon 4 and exon 5, were sequenced from genomic DNA. PCR products were purified with Montage PCR_{96} filter plates (Millipore). Sequencing

reactions (10 µl) contained 2 µl purified PCR product, 1 µl BigDye Terminator Cycle Sequencing Version 3.1 (Applied Biosystems), 1.5 µl of 5x buffer, 5 µl H_2O, and 0.5 µl (1 µM final) of sense or antisense primer. Products were analyzed on an ABI 3730x1 DNA Analyzer (Applied Biosystems). Sequencing files were processed using Sequence Analysis software (Applied Biosystems) and were assembled and analyzed using the Phred/Phrap/Consed System [35, 36]. We have recently reported 42 mutations identified in 204 patients [15].

RESULTS:

Mutation analysis:

Out of the thirty-seven published cases, nine were homozygous for the c.271dupA mutation (patients 6, 7, 11, 12, 14, 17, 18, 27 and 36); this particular mutation has been reported to account for 40% of mutant alleles [15]. Patients 2, 3, 9 and 37 were homozygous for the c.394C>T (p.R132X) mutation. Patients 15, 16 and 20 were homozygous for the c.331C>T (p.R111X) mutation. Two patients (32 and 33) were homozygous for the c.440G>A (p.G147D) mutation. Three patients, 1, 4 and 19, were heterozygous for the c.271dupA and c.394C>T (p.R132X) mutations. Five patients, (5, 8, 21, 22 and 23), were heterozygous for c.271dupA and c.331C>T (p.R111X). Three patients were heterozygous for c.271dupA and c.82-9_12delTTTC (patients 26, 34 and 35). Table 1 summarizes the mutations identified in the thirty-seven patients included in this study.

Phenotype-genotype correlations:

Twenty-five of the thirty-seven (25/37) had early-onset disease, presenting in the first 6 months of life. 17/25 patients were found to be either homozygous for the c.271dupA mutation (n=9) or for the c.331C>T mutation (n=3), or compound heterozygotes for these 2 mutations (n=5). Most of the remaining early-onset patients were either compound heterozygotes for different nonsense mutations, or for a nonsense mutation and a frameshift mutation (see Table 2 for details on types of mutation).

Nine of twelve (9/12) late-onset cases presented with acute neurological symptoms: 4/9 were homozygous for the c.394C>T mutation. These individuals (patients

2, 3, 9 and 37) had clinical courses characterized by normal development and health until early adolescence or adulthood with sudden neurological deterioration (except for the asymptomatic 12-year-old girl identified once the diagnosis was established in her older sister). 2/9 patients with late-onset disease were compound heterozygotes for the c.271dupA and c.394C>T mutations. Interestingly, patient 1, who also was a compound heterozygote for the c.271dupA and c.394C>T, had early-onset disease. 3/9 patients with late-onset disease were compound heterozygotes for the c.271dupA mutation and a missense mutation. The remaining 3/12 patients with late-onset disease had mainly renal involvement in absence of neurological manifestations: all were heterozygous for the c.271dupA mutation and the c.82-9_12delTTTC mutation. This intronic mutation was not seen in any other individual from the database of 204 sequenced patients.

Observations on ethnic background:

Out of the nine published cases homozygous for the c.271dupA mutation, 5 patients are white (of European or North American extraction), one is Hispanic, one Iranian, one Middle Eastern and one with undisclosed ethnic background. From the database, another forty-four patients from all ethnic backgrounds were found to be homozygous for the c.271dupA mutation. Therefore, this mutation was seen in individuals of various backgrounds.

Homozygosity for the c.331C>T (p.R111X) mutation in the published cases was seen exclusively in three Cajun patients (patients 15, 16 and 20). In addition, three unpublished patients of French-Canadian background from the database were also found to be homozygous for this mutation. Compound heterozygosity for the c.331C>T and c.271dupA mutations was identified in 5 published patients: one said to be white from the USA (patient 8), one French Canadian (patient 5), and three from Louisiana, USA (patients 21, 22 and 23). From the database's unpublished patients, an additional five individuals of French-Canadian or Cajun background were found to be heterozygous for the c.271dupA and c.331C>T mutations.

The c.394C>T (p.R132X) mutation, primarily associated with late-onset disease, was noted in homozygous state amongst 4 published individuals of Asiatic-Indian, Pakistani or Middle Eastern descent (patients 2, 3, 9 and 37). Homozygosity for the

c.394C>T mutation was seen in an additional nine unpublished patients from the database, all of whom were either Asiatic-Indian, Pakistani or Middle Eastern. This mutation is seen in heterozygous state along with c.271dupA in two individuals of European ancestry (patients 4 and 19) and one patient of undisclosed ethnic background. In the database, no individual of European ancestry was homozygous for the c.394C>T mutation.

Homozygosity for the c.440G>A (p.G147D) mutation was noted only amongst Native American affected individuals (Patients 32 and 33, and a third non-published case from the original data published by Lerner-Ellis et al).

DISCUSSION:

cblC has been demonstrated to be a heterogeneous condition. Presentation can occur in the neonatal period with multisystemic organ failure in the context of an acute metabolic crisis, by insidious developmental delay without metabolic illness, as a neurological deterioration in a previously well child, adolescence or adult, or as isolated renal disease. With the identification of the MMACHC gene, it has become possible to establish genotype-phenotype correlations, as well as observe ethnic-related trends. While the function of the protein remains to be elucidated, the pathological genetic defect specific to each patient can in part explain the heterogeneity that has come to light since Mudd and Levy's original case report [2, 3].

Differences in RNA stability or residual function of the protein product are predicted to cause, at least in part, differences in phenotypes [37]. In addition, other factors such as background individual genetic variation, environmental and dietary exposures may also account for the variation in presentation in individuals carrying the exact same two mutations. Guigonis et al [13] reported 2 sisters affected with cblC who presented with thrombotic microangiopathy, suggesting haemolytic uremic syndrome, in absence of neurological involvement. The younger of the two sisters had more severe renal disease, associated with renal failure: she was found to be heterozygous for the 3254T>C Factor H polymorphism, which was felt by the authors to explain her more aggressive renal involvement. In addition, the patient reported by Augoustides-Savvopoulou et al [8], who was found to be heterozygous for c.271dupA and c.394C>T,

had onset of the disease after being previously developmentally and neurologically normal: her older sister was reported to have had onset of seizures in the neonatal period, developmental delay, spastic paraparesis, and died at 13 years of age, undiagnosed. It is probable that this child was also affected with cblC and that heterozygosity for c.271dupA and c.394C>T in this family would have led to an early onset case and a late-onset case, demonstrating significant intrafamilial phenotypic heterogeneity in the presence of identical mutations. Another possibility, albeit less likely, is that one of the parents of these girls is a compound heterozygote for both an early onset mutation and the c.394C>T, and the other parent a carrier of the c.271dupA mutation. However, the parent with compound heterozygosity would be expected to be symptomatic: genotyping of the parents would aid in clarifying if this is a case of familial phenotypic heterogeneity or of familial genotypic heterogeneity. Familial genotypic heterogeneity has been reported in the case of PKU and mild benign hyperphenylalaninemia in the same family [38, 39] and could theoretically explain familial variability of phenotype in other metabolic conditions. Some additional factors that help to explain intrafamilial variability may include "synergistic heterozygosity" for disease-associated alleles encoding other enzymes or proteins in the cobalamin pathway, cis polymorphisms affecting folding or residual activity of the mutated enzyme, trans acting factors' interactions with the mutated allele, and non-genetic factors such as diet [40-43].

The mutation c.271dupA accounts for 40% of mutant alleles in our collection of 204 unrelated patients [15]. Upon reviewing the phenotype of patients homozygous for this mutation, the 9 published cases were found to invariably have an early onset (usually within the first 4 months of life), usually with an acute metabolic decompensation. Multisystem organ involvement at the time of diagnosis was not unusual, and most had a progression of their disease despite institution of OHCbl intramuscular (IM) injections. A significant proportion died, and of those who survived the initial presentation, none were cognitively and developmentally normal.

Lerner-Ellis et al noted that patients who are homozygous for the c.394C>T (p.R132X) generally present later in life. The four published individuals with this genotype, including the first late-onset patients reported by Shinnar and Singer, demonstrate this finding. Patient 3 was asymptomatic at the time of diagnosis, whereas

the three other individuals were cognitively and developmentally normal prior to the development of psychiatric and neurological symptoms. In addition, all three symptomatic individuals were responsive to OHCbl IM injections, with complete (or near complete) reversal of the psychiatric and neurological manifestations.

Two patients of the 12 published cases with late-onset disease were found to be compound heterozygous for the c.271dupA (early-onset) and c.394C>T (late-onset) mutations. These individuals did not present with acute metabolic decompensation early in life: both presented earlier than the four patients homozygous for the c.394C>T mutation, and later than the nine patients homozygous for the c.271dupA mutation. After a period of normal development, both individuals manifested acute psychiatric and neurological symptoms. OHCbl therapy reversed most of their symptoms. Therefore, it appears that these compound heterozygotes can manifest a disease intermediate between the severe early-onset form associated with homozygosity for c.271dupA and the late-onset phenotype associated with homozygosity for c.394C>T. However, patient 1, who was also found to be compound heterozygote for these two mutations (as was the older sibling of patient 19, who presented in infancy), presented with a clinical course indistinguishable for the patients homozygous for the c.271dupA mutation, indicating that interpretation of anticipated phenotype based on this genotype may be unreliable.

The presence of c.331C>T in individuals of French Canadian and Cajun ancestry may reflect a case of founder effect and genetic drift. The appearance of this mutation in Cajun individuals may be explained by the Deportation of the Acadians by the British in 1755, where many headed south to settle in Louisiana.

Different mutations at the same nucleotide are noted to produce strikingly different phenotypes, based on the predicted amino acid change. An example of this is demonstrated by the c.440G>A and c.440G>C mutations. The former is expected to cause a glycine to aspartic acid change at codon 147, while the latter is expected to be associated with a glycine to alanine change at the same codon. The c.440G>C (p.G147A) mutation was seen in compound heterozygous state with the c.271dupA mutation, in a patient (patient 24) who presented at the age of 45 years after a normal earlier life, with an unremarkable past medical history. In contrast, the c.440G>A (p.G147D) mutation, in compound heterozygous state with the c.271dupA, was associated with an early onset (all

in the first month of life), and severe systemic involvement in two unpublished patients from the database of 204 sequenced patients. Codon 147 is located in the middle of the putative Cbl binding domain of the protein. Therefore, the substitution glycine by aspartic acid (a bulky, charged amino acid), may greatly disrupt the catalytic ability or conformational state of the protein, resulting in severe early-onset disease. Conversely, replacing glycine for the second smallest, non-polar amino acid alanine theoretically results in a lesser disruption of the protein's integrity and leads to a milder disease manifestation.

Late-onset of disease was seen frequently in patients who are compound heterozygotes for the c.271dupA mutation and a missense mutation: two examples of this from the published cases are those of patient 24 (c.271dupA / c.440G>C, diagnosed at 45 years) and patient 25 (c.271dupA / c.482G>A, diagnosed at 20 years). In fact, adolescent and adult-onset cases were mostly seen in the c.394C>T homozygotes (as discussed above) and in compound heterozygotes for the c.271dupA and a missense mutation.

The c.82-9_12delTTTC mutation (in heterozygous state with the c.271dupA mutation) was only seen in three patients (patients 26, 34 and 35) with the unique presentation of haemolytic uremic syndrome without neurological involvement. Patient 26's younger sibling was also similarly affected. The c.82-9_12delTTTC mutation is a presumed splice site defect. It is possible that this mutation abolishes a particular splice site necessary to generate an MMACHC isoform essential for maintenance of renal integrity. OHCbl injections greatly improved renal function in all 4 patients.

The recognition of apparent phenotype-genotype correlations and the association of mutations with specific ethnicities or populations of origin will be immediately useful for identification of disease-causing mutations in cblC patients, for carrier detection in families where mutations are known, and in setting up initial screening programs in molecular diagnostic labs. There are potential implications for newborn screening: mutation screening tests for the more common early-onset mutations (i.e. c.271dupA and c.331C>T) could be offered in those infants with a positive screen for methylmalonic acid, thus consisting of a second-tier level in identifying cblC pre-symptomatically or early in the course of the disease. Molecular prenatal diagnosis could also be offered to couples at high risk of having affected children.

These findings demonstrate the translation of genomics into the application of a diagnostic tool. Further study into disease mechanism of specific mutations will help to understand phenotypic presentations and the overall pathogenesis in cblC patients.

ACKNOWLEDGEMENTS

In memory of Jamie C. Tirone. The authors would like to thank the referring physicians. We also thank David Watkins, Maria Galvez, Nora Matiaszuk, Gail Dunbar, and Jocelyne Lavallée for technical assistance. Funds from the CIHR (MGP-15078), the March of Dimes Birth Defects Foundation (6-FY01-11) and the Garrod Association contributed to this research. D.S.R. is a principal investigator in the CIHR Group in Medical Genetics. This is a publication of the Hess B. and Diane Finestone Laboratory in Memory of Jacob and Jenny Finestone.

REFERENCE LIST

[1] K.S. McCully, Vascular pathology of homocysteinemia: implications for the pathogenesis of arteriosclerosis. Am. J. Pathol. 56 (1969) 111-128.

[2] S.H. Mudd, H.L. Levy, R.H. Abeles, J.P. Jennedy, Jr., A derangement in B12 metabolism leading to homocystinemia, cystathioninemia and methylmalonic aciduria. Biochem. Biophys. Res. Commun. 35 (1969) 121-126.

[3] H.L. Levy, S.H. Mudd, J.D. Schulman, P.M. Dreyfus, R.H. Abeles, A derangement in B12 metabolism associated with homocystinemia, cystathioninemia, hypomethioninemia and methylmalonic aciduria. Am. J. Med. 48 (1970) 390-397.

[4] D.S. Rosenblatt , W.A. Fenton, Inherited disorders of folate and cobalamin transport and metabolism, in: C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle, B. Childs, K.W. Kinzler (Eds.), The Metabolic & Molecular Bases of Inherited Disease, McGraw-Hill, New York, 2001, pp. 3897-3933.

[5] S. Shinnar , H.S. Singer, Cobalamin C mutation (methylmalonic aciduria and homocystinuria) in adolescence. A treatable cause of dementia and myelopathy. N. Engl. J. Med. 311 (1984) 451-454.

[6] D.S. Rosenblatt, A.L. Aspler, M.I. Shevell, B.A. Pletcher, W.A. Fenton, M.R. Seashore, Clinical heterogeneity and prognosis in combined methylmalonic aciduria and homocystinuria (cblC). J. Inherit. Metab Dis. 20 (1997) 528-538.

[7] G.A. Mitchell, D. Watkins, S.B. Melancon, D.S. Rosenblatt, G. Geoffroy, J. Orquin, M.B. Homsy, L. Dallaire, Clinical heterogeneity in cobalamin C variant of combined homocystinuria and methylmalonic aciduria. J. Pediatr. 108 (1986) 410-415.

[8] P. Augoustides-Savvopoulou, I. Mylonas, A.C. Sewell, D.S. Rosenblatt, Reversible dementia in an adolescent with cblC disease: clinical heterogeneity within the same family. J. Inherit. Metab. Dis. 22 (1999) 756-758.

[9] J.M. Powers, D.S. Rosenblatt, R.E. Schmidt, A.H. Cross, J.T. Black, A.B. Moser, H.W. Moser, D.J. Morgan, Neurological and neuropathologic heterogeneity in two brothers with cobalamin C deficiency. Ann. Neurol. 49 (2001) 396-400.

[10] O.A. Bodamer, D.S. Rosenblatt, S.H. Appel, A.L. Beaudet, Adult-onset combined methylmalonic aciduria and homocystinuria (cblC). Neurology 56 (2001) 1113-

[11] J.L. Van Hove, R. Van Damme-Lombaerts, S. Grunewald, H. Peters, D.B. Van, J.P. Fryns, J. Arnout, R. Wevers, E.R. Baumgartner, B. Fowler, Cobalamin disorder Cbl-C presenting with late-onset thrombotic microangiopathy. Am. J. Med. Genet. 111 (2002) 195-201.

[12] E. Roze, D. Gervais, S. Demeret, B.H. Ogier de, J. Zittoun, J.F. Benoist, G. Said, C. Pierrot-Deseilligny, F. Bolgert, Neuropsychiatric disturbances in presumed late-onset cobalamin C disease. Arch. Neurol. 60 (2003) 1457-1462.

[13] V. Guigonis, V. Fremeaux-Bacchi, S. Giraudier, R. Favier, D. Borderie, Z. Massy, B. Mougenot, D.S. Rosenblatt, G. Deschenes, Late-onset thrombocytic microangiopathy caused by cblC disease: association with a factor H mutation. Am. J. Kidney Dis. 45 (2005) 588-595.

[14] A.L. Boxer, J.H. Kramer, K. Johnston, J. Goldman, R. Finley, B.L. Miller, Executive dysfunction in hyperhomocystinemia responds to homocysteine-lowering treatment. Neurology 64 (2005) 1431-1434.

[15] J.P. Lerner-Ellis, J.C. Tirone, P.D. Pawelek, C. Doré, J.L. Atkinson, D. Watkins, C.F. Morel, T.M. Fujiwara, E. Moras, A.R. Hosack, G.V. Dunbar, H. Antonicka, V. Forgetta, C.M. Dobson, D. Leclerc, R.A. Gravel, E.A. Shoubridge, J.W. Coulton, P. Lepage, J.M. Rommens, K. Morgan, D.S. Rosenblatt, Identification of the gene responsible for methylmalonic aciduria and homocystinuria, cblC type. Nat. Genet. 38 (2006) 93-100.

[16] M.J. Dillon, J.M. England, D. Gompertz, P.A. Goodey, D.B. Grant, H.A. Hussein, J.C. Linnell, D.M. Matthews, S.H. Mudd, G.H. Newns, J.W. Seakins, B.W. Uhlendorf, I.J. Wise, Mental retardation, megaloblastic anaemia, methylmalonic aciduria and abnormal homocysteine metabolism due to an error in vitamin B12 metabolism. Clin. Sci. Mol. Med. 47 (1974) 43-61.

[17] D.W. Bartholomew, M.L. Batshaw, R.H. Allen, C.R. Roe, D. Rosenblatt, D.L. Valle, C.A. Francomano, Therapeutic approaches to cobalamin-C methylmalonic acidemia and homocystinuria. J. Pediatr. 112 (1988) 32-39.

[18] Y. Brandstetter, E. Weinhouse, M.L. Splaingard, T.T. Tang, Cor pulmonale as a complication of methylmalonic acidemia and homocystinuria (Cbl-C type). Am. J. Med. Genet. 36 (1990) 167-171.

[19] P.B. Kazimiroff , D.M. Shaner, Methylmalonic acid and homocystinuria (cobalamin C mutant disease) presenting as acute paraparesis in an adolescent. Ann. Neurol. 30 (1991) 468-468.

[20] F.A. Wijburg, D.S. Rosenblatt, G.D. Vos, J.W. Oorthuys, L.G. van't Hek, B.J. Poorthuis, M.K. Sanders, R.B. Schutgens, Clinical and biochemical observations in a patient with combined Pompe disease and cblC mutation. Eur. J. Pediatr. 151 (1992) 127-131.

[21] M.T. Geraghty, E.J. Perlman, L.S. Martin, S.J. Hayflick, J.F. Casella, D.S. Rosenblatt, D. Valle, Cobalamin C defect associated with hemolytic-uremic syndrome. J. Pediatr. 120 (1992) 934-937.

[22] P. Russo, J. Doyon, E. Sonsino, H. Ogier, J.M. Saudubray, A congenital anomaly of vitamin B12 metabolism: a study of three cases. Hum. Pathol. 23 (1992) 504-512.

[23] R. Howard, I.J. Frieden, D. Crawford, T. McCalmont, M.L. Levy, D.S. Rosenblatt, L. Sweetman, S.I. Goodman, C. Ohnstad, K. Hart, M. Berrios, S. Packman, Methylmalonic acidemia, cobalamin C type, presenting with cutaneous manifestations. Arch. Dermatol. 133 (1997) 1563-1566.

[24] H.C. Andersson , E. Shapira, Biochemical and clinical response to hydroxocobalamin versus cyanocobalamin treatment in patients with methylmalonic acidemia and homocystinuria (cblC). J. Pediatr. 132 (1998) 121-124.

[25] C. Ellaway, J. Christodoulou, R. Kamath, K. Carpenter, B. Wilcken, The association of protein-losing enteropathy with cobalamin C defect. J. Inherit. Metab Dis. 21 (1998) 17-22.

[26] B. Merinero, C. Perez-Cerda, M.J. Garcia, B. Chadefaux-Vekemans, P. Kamoun, C. Tonetti, J. Zittoun, C. Jakobs, M. Ugarte, Reliability of biochemical parameters used in prenatal diagnosis of combined methylmalonic aciduria and homocystinuria. Prenat. Diagn. 18 (1998) 947-952.

[27] G.M. Enns, A.J. Barkovich, D.S. Rosenblatt, D.R. Fredrick, K. Weisiger, C. Ohnstad, S. Packman, Progressive neurological deterioration and MRI changes in cblC methylmalonic acidaemia treated with hydroxocobalamin. J. Inherit. Metab Dis. 22 (1999) 599-607.

[28] H.C. Andersson, M. Marble, E. Shapira, Long-term outcome in treated combined methylmalonic acidemia and homocystinemia. Genet. Med. 1 (1999) 146-150.

[29] C.O. Harding, D.A. Pillers, R.D. Steiner, T. Bottiglieri, D.S. Rosenblatt, J. Debley, G.K. Michael, Potential for misdiagnosis due to lack of metabolic derangement in combined methylmalonic aciduria/hyperhomocysteinemia (cblC) in the neonate. J. Perinatol. 23 (2003) 384-386.

[30] P.J. Francis, D.M. Calver, P. Barnfield, C. Turner, R.N. Dalton, M.P. Champion, An infant with methylmalonic aciduria and homocystinuria (cblC) presenting with retinal haemorrhages and subdural haematoma mimicking non-accidental injury. Eur. J. Pediatr. 163 (2004) 420-421.

[31] R. Heidenreich, T. Grebe, M. Colville, K. Aleck, cblC defect in three Southwestern Native American infants. Mol. Genet. Metab. 84 (2005) 223-223.

[32] E.K. Tsina, D.L. Marsden, R.M. Hansen, A.B. Fulton, Maculopathy and retinal degeneration in cobalamin C methylmalonic aciduria and homocystinuria. Arch. Ophthalmol. 123 (2005) 1143-1146.

[33] D.S. Rosenblatt, B.A. Cooper, A. Pottier, H. Lue-Shing, N. Matiaszuk, K. Grauer, 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 (1984) 2149-2156.

[34] D. Watkins, N. Matiaszuk, D.S. Rosenblatt, Complementation studies in the cblA class of inborn error of cobalamin metabolism: evidence for interallelic complementation and for a new complementation class (cblH). J. Med. Genet. 37 (2000) 510-513.

[35] B. Ewing, L. Hillier, M.C. Wendl, P. Green, Base-calling of automated sequencer traces using phred. I. Accuracy assessment. Genome Res. 8 (1998) 175-185.

[36] D. Gordon, C. Abajian, P. Green, Consed: a graphical tool for sequence finishing. Genome Res. 8 (1998) 195-202.

[37] L.E. Maquat, Nonsense-mediated mRNA decay:splicing, translation and mRNP dynamics. Nat. Rev. Mol. Cell Biol. 5 (2004) 89-99.

[38] F.D. Ledley, H.L. Levy, S.L. Woo, Molecular analysis of the inheritance of phenylketonuria and mild hyperphenylalaninemia in families with both disorders. N. Engl. J. Med. 314 (1986) 1276-1280.

[39] P. Guldberg, H.L. Levy, R. Koch, C.M. Berlin, Jr., B. Francois, K.F. Henriksen, F. Guttler, Mutation analysis in families with discordant phenotypes of phenylalanine hydroxylase deficiency. Inheritance and expression of the hyperphenylalaninaemias. J. Inherit. Metab. Dis. 17 (1994) 645-651.

[40] J. Vockley, P. Rinaldo, M.J. Bennett, D. Matern, G.D. Vladutiu, Synergistic heterozygosity: disease resulting from multiple partial defects in one or more metabolic pathways. Mol. Genet. Metab. 71 (2000) 10-18.

[41] C.R. Scriver , P.J. Waters, Monogenic traits are not simple: lessons from phenylketonuria. Trends Genet. 15 (1999) 267-272.

[42] K.M. Dipple, J.K. Phelan, E.R. McCabe, Consequences of complexity within biological networks: robustness and health, or vulnerability and disease. Mol. Genet. Metab. 74 (2001) 45-50.

[43] K.M. Dipple , E.R. McCabe, Modifier genes convert "simple" Mendelian disorders to complex traits. Mol. Genet. Metab. 71 (2000) 43-50.

TABLES

Table 1: Clinical and molecular data on published patients, listed in order of year of publication. Pt=Patient, Asymp=Asymptomtic, [a]Siblings, [b]Consanguinity

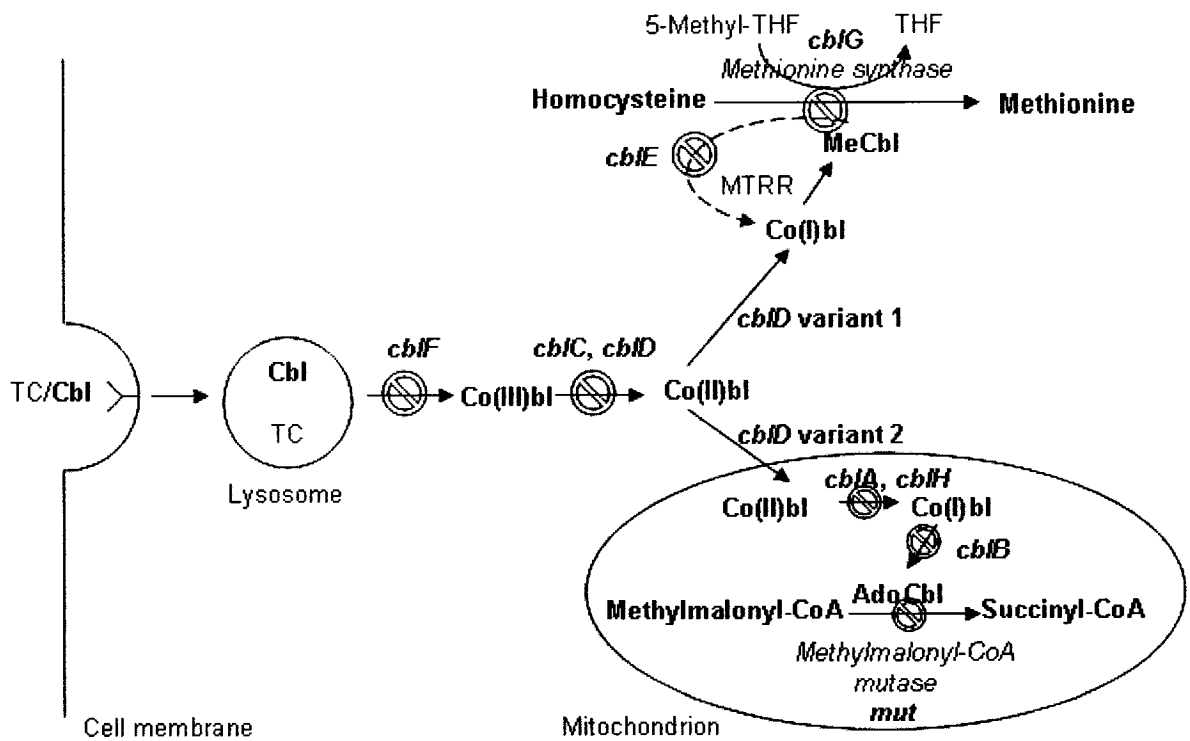
Pt	Reference	Sex	Onset	Ethnicity	Mutation 1	Mutation 2
1	Dillon et al.[16]	F	Early	?	c.271dupA	c.394C>T
[a]2	Shinnar, Singer[5]	F	Late	Asiatic-Indian	c.394C>T	c.394C>T
[a]3		F	Asymp	Asiatic-Indian	c.394C>T	c.394C>T
4	Mitchell et al.[7]	M	Late	Portuguese	c.271dupA	c.394C>T
5		F	Early	French-Canadian	c.271dupA	c.331C>T
6	Bartholomew et al.[17]	M	Early	White	c.271dupA	c.271dupA
7		F	Early	White	c.271dupA	c.271dupA
8	Brandstetter et al.[18]	M	Early	White	c.271dupA	c.331C>T
9	Kazimiroff, Shaner[19]	F	Late	Asiatic-Indian	c.394C>T	c.394C>T
10	Wijburg et al.[20]	M	Early	[b]Turkish	c.80A>G	c.80A>G
11	Geraghty et al[21]	F	Early	White	c.271dupA	c.271dupA
12	Russo et al.[22]	M	Early	French-Canadian	c.271dupA	c.271dupA
13	Howard et al.[23]	F	Early	Hispanic	c.331C>T	c.615C>G
14		F	Early	Hispanic	c.271dupA	c.271dupA
15	Andersson, Shapira[24]	M	Early	White-Louisiana	c.331C>T	c.331C>T
16		F	Early	Cajun	c.331C>T	c.331C>T
17	Ellaway et al.[25]	M	Early	[b]Middle Eastern	c.271dupA	c.271dupA
18	Merinero et al.[26]	F	Early	White-Spain	c.271dupA	c.271dupA
13	Enns et al.[27]	See details on patient 13 published by Howard et al.[23]				
19	Augoustides-S. et al[8]	F	Late	Greek	c.271dupA	c.394C>T
15	Andersson et al.[28]	See details on patient 15 published by Andersson and Shapira[24]				
20		F	Early	Cajun	c.331C>T	c.331C>T
16		See details on patient 16 published by Andersson and Shapira[24]				
21		M	Early	White-Louisiana	c.271dupA	c.331C>T
22		M	Early	White-Louisiana	c.271dupA	c.331C>T
23		F	Early	White-Louisiana	c.271dupA	c.331C>T
24	Powers et al.[9]	M	Late	White	c.271dupA	c.440G>C
25	Bodamer et al.[10]	M	Late	Hispanic	c.271dupA	c.482G>A
26	Van Hove et al.[11]	M	Late	White	c.82-9_12delTTTC	c.271dupA
27	Harding et al.[29]	M	Early	[b]Middle Eastern	c.271dupA	c.271dupA
28		F	Early	Hispanic	c.328_331delAACC	c.328_331delAACC
29	Roze et al.[12]	F	Late	Middle Eastern	c.271dupA	c.347T>C
30	Francis et al.[30]	F	Early	Asian	c.457C>T	c.481C>T
31	Heidenreich et al.[31]	F	Early	Native Amercican	c.328_331delAACC	c.394C>T
32		F	Early	Native American	c.440G>A	c.440G>A
33		M	Early	Native American	c.440G>A	c.440G>A
[a]34	Guigonis et al.[13]	F	Late	White – France	c.82-9_12delTTTC	c.271dupA
[a]35		F	Late	White – France	c.82-9_12delTTTC	c.271dupA
36	Tsina et al.[32]	F	Early	?	c.271dupA	c.271dupA
37	Boxer et al.[14]	M	Late	[b]Middle Eastern	c.394C>T	c.394C>T

Table 2: Mutations and predicted effect on protein or mRNA

DNA	PROTEIN	TYPE OF MUTATION
c.3G>A	p.Met1?	initiation codon change
c.80A>G	p.Gln27Arg	missense
c.82-9_12delTTTC	r.spl?	splice site
c.271dupA	p.Arg91LysfsX14	frameshift-insertion
c.328_331delAACC	p.Asn110AspfsX13	frameshift-insertion
c.331C>T	p.Arg111X	nonsense
c.347T>C	p.Leu116Pro	missense
c.394C>T	p.Arg132X	nonsense
c.440G>A	p.Gly147Asp	missense
c.440G>C	p.Gly147Ala	missense
c.457C>T	p.Arg153X	nonsense
c.481C>T	p.Arg161X	nonsense
c.482G>A	p.Arg161Gln	missense
c.615C>G	p.Tyr205X	nonsense

FIGURES

Figure 1: Summary of inborn errors of Cbl metabolism, including TC deficiency and mutase deficiency. AdoCbl= Adenosylcoblamin, Cbl= Cobalamin, *cblA-cblG*= Cbl complementation group diseases, MeCbl= Methylcobalamin, MTRR= Methionine synthase reductase, mut= Methylmalonyl-CoA mutase deficiency, TC/Cbl= Transcobalamin-cobalamin complex, TC= Transcobalamin, THF= Tetrahydrofolate



CHAPTER 6.0: GENERAL DISCUSSION

6.0.1 Lessons from the identification and characterization of the *MMAA* gene:

In chapter 2, the identification of the *MMAA* gene is described in detail. The immediate consequence of this work was the ability to identify mutations in patients affected with *cblA* disease. My work has been to identify and characterize mutations in the *MMAA* gene and to try to relate this to disease mechanism and clinical presentation. Thirty-seven patients were screened for mutations in the *MMAA* gene and 19 novel mutations were identified. Diagnostic tests were designed for all 22 known mutations. This information was immediately disseminated to the physicians who had treated these patients with *cblA* disease to allow for genetic counseling and the opportunity for prenatal diagnoses in families where mutations were identified.

One mutation, c.433C>T (p.R145X) accounted for 43% of pathogenic alleles and was identified on a unique haplotype. A noncoding SNP (c.820-110A>G) was observed in linkage disequilibrium only with c.433C>T and was not observed at all in control DNA. Control samples from the CEU (CEPH, Utah) cohort were used, and these represent individuals of European ancestry and match those individuals who carry the c.433C>T mutation. Notably, most individuals with c.433C>T were of European ancestry, except one who was reported as White and Asian and one who was Black. These findings suggest that patients that carry the c.433C>T mutation, may have inherited it from a common ancestor, and the c.820-110A>G SNP may be considered as linked to the causal mutation. It would be interesting to extend the haplotype in these individuals to evaluate the extent of linkage disequilibrium and the age of this mutation, but these studies are limited by the lack of cell lines (or DNA) from family members to properly phase affected individuals. Extending haplotypes in individuals homozygous for a mutation might provide some insight into the history of these mutations by looking at the extent of homozygous stretches. Longer stretches indicate a more recent mutation event or a more recent founder mutation. The c.433C>T mutation was observed in another study in heterozygous form in one individual reported as having Asian ancestry (227). It would be interesting to see if this mutation occurs on the same haplotype background as individuals who carry this mutation from our collection.

Another mutation identified by Yang *et al* was c.503delC and accounted for 57% of alleles in their collection (227). This mutation was observed in heterozygous state in one individual from our collection of patients and who was reported as having White and Asian ancestry. Other mutations identified by Yang *et al* included R22X, and novel mutations L217X and R359G (227). Further study into haplotypes will allow for the determination of recurrent versus ancestral mutation events. Nine of twenty-two mutations were observed in exon 2. The sequence content of exon 2 alone could not explain this clustering. Two mutations in this exon, c.64C>T (R22X) and c.433C>T (R145X) do occur at CpG sites which are prone to mutation.

In our laboratory, c.433C>T was observed in homozygous form in ten patients who presented from one to ten years in life and environmental factors and or genetic modifiers were suggested as a possible explanation for these different ages of onset. Another mutation, c.592_595delACTG was also common and observed in six different individuals. One missence mutation (Q363H) was identified only in four of one hundred controls (4%) and may or may not be implicated in the metabolism of methylmalonic acid in the general population. Cell culture studies on individuals with this sequence change may or may not help clarify this issue. Mass spectroscopy and gas chromatography of plasma and urine could also be used to detect elevated levels of methylmalonic acid. Molecular characterization of the *MMAA* gene has provided the foundation for further study into the function of this gene product.

6.0.2 Lessons learned following the identification and characterization of mutations in the MMAB gene:

Sequence analysis of gDNA from a panel of *cblB* patient fibroblasts was used to identify 13 novel mutations. A total of 19 mutations were identified, most (11/19) occurring in exon 7, suggested to be a mutation hotspot due to high CpG rich content as well as repetitive sequence content. Notably, exon 7 encodes part of the enzyme active site of the adenosyltransferase and is well conserved among multiple species including eukaryotes, bacteria and archea. The most common mutation in *cblB* patients was c.556C>T (p.R186W), accounting for 33% of pathogenic alleles in our cohort; it occurs at the active site of the enzyme and was shown to eliminate enzyme activity in cob(I)alamin

adenosyltransferase from *Thermophilus acidophilum in vitro*. In patients it was associated with severe disease, with presentation in the first year of life. It was observed on a common haplotype that was also observed frequently in control populations. Because c.556C>T occurs at a potential mutation hotspot, it is possible that the frequency of this mutation is the result of more than one mutation event on a common haplotype. It will be necessary to extend this haplotype and examine the extent of linkage disequilibrium to evaluate this hypothesis.

The recent determination of the crystal structure of a PduO cobalamin adenosyltransferase from *Thermophilus acidophilum*, which is 32% identical to human MMAB, provided insight into the nature of adenosyltransferase function (228). At least seven different missense mutations identified in *cblB* patients occur at the enzyme active site. Verification of the effect of these mutations on enzyme function using *in vitro* enzymology studies will be necessary.

Only one nonsense mutation was identified (c.700C>T [p.Q234X]) in our cohort of *cblB* patients. This sequence change was associated with higher levels of MCM function than in other *cblB* fibroblast cell lines in cell culture studies (in homozygous form or in compound heterozygous form with another mutation) but was not correlated with reduced severity of clinical presentation. The c.197-1G>T mutation was associated with a severe cellular phenotype but patients homozygous for this mutation had a later age of onset. These findings make it difficult to clearly correlate mutations in *MMAB* with clinical phenotype. As was noted in *cblA* disease, phenotype genotype correlations in *cblB* disease may be influenced by the presence of genetic modifiers or differences in environmental conditions. The *cblB* disorder generally has a more severe clinical course and is less responsive to therapy then the *cblA* disorder. However, both disease groups are more responsive to cobalamin supplementation then are other inborn errors of cobalamin metabolism.

6.0.3 Lessons learned from the identification of the MMACHC gene:

Multiple strategies were used in the identification of the *MMACHC* gene. These included a linkage study of six families, sibling pair analysis, homozygosity mapping, and haplotype analyses. Furthermore, a complete draft sequence of the human genome

combined with data availability and bioinformatics tools allowed for selection of candidate genes based on ESTs, expression, functional domain and gene conservation profiling. Only with the combination of all these strategies were we able to identify the *MMACHC* gene. Known pitfalls in all of these methods made for increased complexity of the challenge.

The analysis of six families informative for linkage was successful in narrowing the chromosomal interval to the short arm of chromosome 1. Sibling pair analysis confirmed the authenticity of the linkage study and confirmed the localization of the MMACHC gene to a 6.6 Mb interval. Homozygosity mapping allowed for further refinement of the chromosomal interval to ~2 Mb. This, followed by screening of candidate genes, and the identification of common SNPs and of shared haplotypes refined the region to a shorter interval. Four causal mutations were identified in a previously uncharacterized EST: AL080062 in DNA from eight *cblC* individuals.

We reported 42 mutations in 204 *cblC* individuals. As described in Chapter 4, c.271dupA accounted for 40% of all pathogenic alleles. This mutation occurred primarily on a common haplotype and was suggested to have a common founder with mutation and recombination having occurred on the same haplotype background subsequent to the original mutation event. However, the c.271dupA mutation occurs on the second most common haplotype identified in CEPH controls making it difficult to definitively conclude a single common founder for this mutation. An identical genotype constructed from two microsatellite markers directly flanking the *MMACHC* gene, D1S2677 (179 kb telomeric to *MMACHC*) and D1S2175 (47 kb centromeric to *MMACHC*), was identified in most *cblC* individuals homozygous for the c.271dupA mutation. To further evaluate this finding, additional genotyping work extended the haplotype out 5' and 3' by 1.0 kb and 4.5 kb respectively using two known SNPs, rs6429566 & rs2993263, which act as tag SNPs for haplotype blocks adjacent to the 10 Kb of gDNA that encompases the *MMACHC* gene. These data are suggestive of an ancestral mutation event. Further analysis by increasing the density of markers and extending the haplotypes in individuals homozygous for the c.271dupA mutation will be necessary to evaluate the extent of linkage disequilibrium and the history of this mutation.

The c.811A>G sequence change was observed on only three chromosomes carrying the c.331C>T mutation. c.811A>G is likely in cis with c.331C>T since it is seen in individuals homozygous for c.331C>T. New data from the international HapMap project (www.hapmap.org) demonstrated that c.811A>G is a known polymorphism (rs35219601). However, it has only been observed in individuals of Yoruban descent, from Sub-Saharan Africa; it is not seen in individuals typed of Hispanic, African American, European or Asian descent. This sequence change results in an amino acid change of p.S271G. This finding suggests that either c.331C>T or c.811A>G may have occurred more than once in history. It is not clear whether or not c.811A>G occurred after the original c.331C>T mutation event but it is possible that c.811A>G modified the ancestral haplotype. Since c.331C>T was demonstrated to occur at a CpG site and is seen on more than one haplotype background, it is also possible that c.331C>T recurred on a genetic background containing the c.811A>G variant. Further genotyping studies will be necessary to evaluate this hypothesis. The observation that the c.811A>G sequence change occurs at a frequency of ~0.545 in the Yoruban panel and that it is only seen in heterozygous state in those genotyped suggests the possibility that it may result in a change in the catalytic activity of the protein product. This is interesting since there are implications for elevated or decreased levels of homocysteine in these individuals. By obtaining cell lines carrying this sequence change from the Yoruban panel population of the HapMap project and studying their ability to incorporate labeled 5'methylTHF into cellular macromolecules, the effect of c.811A>G could be determined. It will also be important to determine the frequency of this sequence change in other populations.

Seven *MMACHC* mutations occur at potential CpG mutation hotspots and four mutations may have occured due to slippage during DNA replication. Of the more common mutations that were observed frequenctly in homozygous form, including c.331C>T (8% of mutant alleles) and c.394C>T (8% of mutant alleles), the identification of these mutations on at least two haplotype backgrounds is suggestive of recurrent mutation and of a mutation hotspot. It would be interesting to see if these residues are consistently methylated in different species throughout evolution.

The c.331C>T and c.271dupA mutations were associated with early onset disease and c.394C>T was associated with late onset disease. Differences in RNA stability or

residual function of the protein product were predicted to cause differences in phenotypes. It is currently not known what the underlying mechanism for these differences are. Nonsense mediated RNA decay is one possible mechanism that may explain these observations. Further experimentation is necessary to evaluate this hypothesis.

The C-terminal end of the *MMACHC* gene was not well conserved in eukaryotes outside mammalia. However, sequence analysis and molecular modeling demonstrated that the C-terminal end was homologous to part of a bacterial protein, TonB. The C-terminal end of *MMACHC* was shown to fold similarly to TonB, a protein involved in the transduction of energy derived from the proton motive force to the transport of cobalalmin and other molecules across the periplasm. The absence of an *MMACHC*-like gene in bacteria suggests a possible gap in evolution. If the C-terminal domain has evolved in mammals to accomadate functions similar to TonB in bacteria, this is a striking example of convergent evolution. The similarities of the TonB-like MMACHC domain to TonB from bacteria suggests it may function in a similar manner to bacterial protein; in this case it would make direct contact with a cognate protein partner to facilitate conformational changes necessary for some aspect of cobalamin metabolism. We suggest that MMACHC intereacts with a lysosomal transmembrane protein, a mitochondrial protein, or both, and may act as a chaperone to shuttle cobalamin in the cell. Cellular colocalization studies are currently underway to test this hypothesis and preliminary data suggests that MMACHC may colocalize to the lysosomal compartment. The MMACHC protein has been purified to homogeneity and surface plasmon resonance technology is being used to try to identify potential protein-binding partners.

Sequence with identity to the canonical cobalamin-binding motif was identified in the MMACHC gene and was conserved in all species examined (221). Cobalamin is proposed to bind MMACHC, which can then be shuttled between cellular compartments. Protein purification and enzymological assays will be necessary to test this hypothesis.

The clinical manifestations in *cblC* disease are well documented but only poorly understood. While neurological findings can be attributed to improper DNA synthesis, severe retardation of myelination in the nervous system in part due to reduced levels of the activated form of methionine, S-adenosylmethionine, as well as decreased

neurotransmitter production, there is little evidence demonstrating the pathological mechanism of this process. Recently, subacute combined degeneration of the spinal cord was seen in a patient with *cblC* disease. This individual had multifocal demyelination, with low plasma methionine levels (229). Experimental studies in animals have supported the theory of hypomethioninemia resulting in SCD. Nitrous oxide in monkeys and pigs inhibits methionine synthase and produces lesions analogous to SCD in humans. Both species had impairment of remethylation and reduced methionine. When methionine was supplemented in the diet prior to nitrous oxide exposure, the spinal cord was fully or partially protected from SCD (230,231).

There is very little known about the classic "salt and pepper" pigmentary retinopathy observed in many *cblC* patients. At least 11 cases of ophthalmic descriptions have been published and reviewed by Schimel & Mets (232). Retinal degeneration has been shown definitively by electroretinogram recordings (232). It was suggested that decreased levels of glutathione (GSH) as the result of depletion of methionine, which normally serves as a methyl donor for the cysteine residue in GSH, might result in lack of protection agasinst oxidative injury and may explain the progressive retinal degeneration in *cblC* disease (232,233). It has been suggested that increased thromboses in patients with *cblC* may lead to ischemic lesions in the eye, but this does not explain why other forms of cobalamin metabolism resulting in combined homocystinuria such as *cblF* and *cblD* do not present with similar findings. Another suggested explanation involved patterns of tissue specific expression of *MMACHC*. However, based on northern blot analysis and evaluation of EST databases, *MMACHC* is ubiquitously expressed in humans with slightly higher levels of transcript detected in fetal liver (221). Interestingly two sisters with a splice site mutation, c.82-9_12delTTTC, presented with thrombotic microangiopathy, suggesting haemolytic uremic syndrome without neurological involvement. One sister who had more severe renal disease was heterozygous for a factor H polymorphism (3254T>C) and this was suggested to explain the more aggressive renal involvement. It is possible that the c.82-9_12delTTTC mutation abolishes a splice site necessary to generate an *MMACHC* isoform necessary for maintenance of renal integrity. Further understanding of MMACHC expression patterns during development and in different tissues will be necessary to understand the pathogenesis of disease in these

patients. The creation of a mouse model of *cblC* disease is currently underway using an embryonic stem cell *MMACHC* gene trapped clone. A mouse model may allow for careful characterization of *MMACHC* expression patterns during rodent development. If mice present with a phenotype similar to *cblC* patients it may also be possible to further dissect some aspects of disease mechanism such as pigmentary retinopathy.

Retrovirus mediated infection of wild-type *MMACHC* into *cblC* patient fibroblasts demonstrated correction of the cellular phenotype. This was achieved by measuring the incorporation of [^{14}C]methylTHF and [^{14}C]propionate incorporation into cellular macromolecules to indirectly measure the activity of methionine synthase and methylmalonyl CoA mutase respectively. Constitutive expression of MMACHC in these cell lines resulted in restoration of these measures to control levels or above. Synthesis of cobalamin derivatives was also measured and infection of MMACHC into patient fribroblasts restored these derivatives to normal levels. Interestingly, the intracellular level of unmetabolized CNCbl was decreased in both *cblC* and control fibroblasts suggested that CNCbl was recruited into cellular metabolism more effectively. Of note, *cblC* individuals respond better to therapy with OHCbl than CNCbl. These observations suggest that a defect in MMACHC may decrease decyanation of CNCbl and/or reduction of Cbl either directly or indirectly. Homology searches suggest that one domain may have weak similarity to an iron-sulphur reductase but further investigation into this hypothesis is necessary to confirm reductase activity.

We have learned a great deal from identification of the gene responsible for the *cblC* inborn error of cobalamin metabolism. It is now possible to implement molecular diagnostic strategies in families where mutations are known, and screening for common mutations as a diagnostic strategy in patients suspected of having the *cblC* disorder. Careful evaluation of control populations and increased awareness on the part of physicians will be necessary to assess the true prevalence of this disease. Evaluation of heterozygous advantage or disadvantage of mutations such as c.271dupA or c.394C>T is also an important question that requires more study. Moreover, rare or common sequence variants in this gene such as the c.800G>A sequence change, observed at high frequency only in the Yoruban population, have implications for differences in the cellular metabolism of this vitamin. The association of cobalamin deficiency with

hyperhomocysteinemia, which is associated with a large number of common complex diseases such as birth defects (neural tube defects, and possibly cleft lip and Down syndrome), psychiatric disease, dementia and Alzeimer disease as well as cardiovascular disease (including stroke) and cancer means that further study into effects of MMACHC polymorphisms on these diseases has important implications. To date, these have been associated with hyperhomocysteinemia or folate deficiency, but the role of cobalamin in conversion of homocysteine to methionine suggests a probable role for cobalamin level in regulation of homocysteine levels. The dissection of *cblC* disease and characterization of binding partners and specific cellular processes as well as the pathogenesis of disease in mice will be important to understanding Cbl metabolism in general.

6.1 A MODEL FOR COBALAMIN UTILIZATION

Cobalamin has been described as a "supernucleophile" and is considered one of the most reactive nucleophiles known in biology (234). For this reason cobalamin likely resorts to the use of chaperones to shelter reactive coenzymes from incidental side reactions and to escort them to dependent enzymes. To cope with the challenge of acquiring this low abundant vitamin in nature, bacteria have evolved a highly efficient active transport system to import cobalamin into the cell. Similarly, humans have also evolved a complex enzyme system for the absorption and transport of cobalamin in the blood and delivery of this scarce nutrient to cells for utilization in key metabolic processes. Estimated concentrations range from 0.03-0.7 uM depending on the organ (170). Cobalamin is delivered to cells in the "base-on" conformation suggested by the crystal structure of TC-bound cobalamin (85). Cobalamin is delivered to the lysosome by lysosome mediated endocytosis, TC is degraded and cobalamin is free. It has been proposed that the acidic pH environment of the lysosome may increase the "base-off" species and that cobalamin exits the lysosome in this conformation through an unidentified transporter that is defective in *cblF* patients (170,60,61). We propose that MMACHC may bind this unidenfied lysosomal transporter to facilitate conformational changes necessary for cobalamin transport into the cytoplasm. MMACHC then binds cobalamin in the "base-off" conformation and acts as a chaperone and either interacts with the as yet unidentified *cblD* product where it undergoes additional processing.

Alternatively, cobalamin may be passed to the *cblD* product or facilitate transport of Cbl directly to mitochondrial and cytoplasmic compartments. Mutations of the *cblD* product have recently been shown to be involved in combined and isolated forms of methylmalonic aciduria and homocystinuria, and may ultimately be responsible for the fate of cobalamin in the cell (66). Delivery of Cbl by the *cblD* product to methionine synthase and to adenosyltransferase is a possible explanation for these observations in patient cell lines.

It is not clear how cobalamin enters the mitochondria. This process may or may not involve a specific transport process. Passive transport has been suggested but this process has also been demonstrated to have some specificity (235,107). One might speculate that the *cblD* product may act as a mitochondrial transporter and also interact with cytoplasmic enzymes involved in cobalamin metabolism. One proposal is that the three mitochondrial proteins in this pathway exist as a complex (170). The longstanding belief is that cobalamin is delivered to the mitochondria in the cob(II)alamin oxidation state where it may bind MMAB. This might be facilitated by the G-protein, MMAA but there is currently no known mechanism. MMAA may then gate the release of AdoCbl from MMAB and transfer AdoCbl directly to MCM and protect AdoCbl from oxidative inactivation. MMAA is currently thought to protect the radical enzyme, MCM, from inactivation and this may be facilitated by GTP hydrolysis (170). MCM and cobalamin, in the "base-off" conformation, then facilitates the isomerization of L-methylmalonyl CoA to succinyl CoA and the free radical form of cobalamin is protected by the MCM/MMAA complex to carry out this reaction. In contrast to the reductive methylation cycles carried out by MS and its partner MSR, MCM and MMAA employ a protective or preventative strategy to protect MCM from inactivation.

6.2 CONCLUSION & SUMMARY

The keen observations of clinicians in the last century led to the identification of a group of diseases that were attributed to defects in the metabolism of vitamin B_{12}. Individuals affected with these diseases usually present very early in life with very severe life threatening symptoms. In the last three decades, the careful collection and preservation of patient cell lines has provided scientists with a tremendous resource for the study of these diseases. Complementation studies led to the classification of eight different disease classes. To date, almost all of the genes responsible for these diseases have been identified using a combination of knowledge obtained from the study of cobalamin metabolism in bacteria and reverse genetic studies. The identification and characterization of the genetic basis of disease has been the principle objective of this laboratory. These studies will lead to improved knowledge of the cellular metabolism of homocysteine and methylmalonic acid as well as the cellular metabolism and fate of vitamin B_{12}.

The recent identification of the genes responsible for *cblA* and *cblB* disease (*MMAA* and *MMAB* respectively) groups opened up several avenues of research including, the molecular genetic dissection of these diseases and the study of the functional properties of these gene products. This thesis focused on researching the genetic basis of disease in these patient cell lines and attempting to understand genotype-phenotype correlations as well as the mechanism of disease mechanism and population genetics.

Advances into our understanding of the genetic basis of disease in *cblC* individuals came with the collection of families and patient samples. A linkage study and homozygosity mapping studies helped to narrow the region responsible for *cblC* disease and was the basis of further investigation into the molecular basis of this disease. Linkage analysis, homozygosity mapping, sibling pair and haplotype analyses were all used to ultimately identify the gene responsible for *cblC* disease, called *MMACHC*, a member of a novel gene family. We demonstrated that mutations in the *MMACHC* gene cause *cblC* disease. In addition we learned about the history of many of the mutations identified in patients by analyzing haplotypes; the large number of patients with common mutations allowed for the identification of specific genotype phenotype correlations; several

domains were identified in the protein by searching protein databases in combination with molecular modeling helped to determine functional aspects of the gene product, including a vitamin B_{12} binding domain and a TonB-like domain.

This work has led to our improved understanding of vitamin B_{12} metabolism. Biophysical studies on *MMAA* and *MMAB* continue to provide insight into disease mechanism and protein function. The identification of the *MMACHC* gene has led to additional studies in our laboratory including, the expression and purification of the protein product, searches for potential binding partners involved in vitamin B_{12} metabolism using surface plasmon resonance technology, functional studies on binding of vitamin B_{12} to MMACHC, cellular colocalization, investigation of the mechanism of late onset disease, and the development of a mouse model.

6.3 CLAIMS TO ORIGINALITY

The following are original contributions to the knowledge of the genetics of vitamin B_{12} metabolism.

Chapter 2.1:

Identification of 18 novel mutations in the *MMAA* gene in *cblA* patients, eight of which were common to two or more individuals. Description of the spectrum of mutations in *cblA* patients and recognition of a common mutation: c.433C>T representing 43% of pathogenic alleles on a common haplotype genetic background.

Chapter 3.1:

Identification of 13 novel mutations in the *MMAB* gene in *cblB* patients. Description of the spectrum of mutations in *cblB* patients. Identification of a common mutation: c.556C>T which accounted for 33% of pathogenic alleles, associated with disease presentation in the first year of life, and was observed on a common haplotype.

Chapter 4.1:

The discovery of the gene responsible for the *cblC* inborn error of vitamin B_{12} metabolism called *MMACHC* and proof that mutations in this gene cause *cblC* disease. Identification of forty-two different novel mutations in 204 patient samples. Identification of three mutations occurring at elevated frequency: c.271dupA, c.331C>T and c.394C>T. Recognition that the c.271dupA and c.331C>T mutations were associated with early onset disease and that c.394C>T was associated with late onset disease. The identification of specific haplotypes for different mutations. Recognition of sequence identity between human MMACHC and bacterial protein TonB and demonstration by molecular modeling that C-terminal region of MMACHC folds similarly to TonB from *E. coli* suggesting a possible role for the MMACHC gene product.

Chapter 5.0:

Further delineation of the relationship between genotype and phenotype in the *MMACHC* gene.

BIBLIOGRAPHY

1. Rosenblatt, D. S. and W. A. Fenton. 2001. Inherited disorders of folate and cobalamin transport and metabolism. In The Metabolic and Molecular Bases of Inherited Disease. C. R. Scriver, A. L. Beaudet, W. S. Sly, D. Valle, B. Childs, K. W. Kinzler, and B. Vogelstein, editors. McGraw-Hill, New York. 3897-3933.

2. Stabler, S. P. 1999. B_{12} and nutrition. In Chemistry and Biochemistry of B_{12}. R. Banerjee, editor. John Wiley & Sons, New York. 343-365.

3. Kratky, C. and B. Krautler. 1999. X-ray crystallography of B_{12}. In Chemistry and Biochemistry of B_{12}. R. Banerjee, editor. John Wiley & Sons, New York. 9-41.

4. Hodgkin, D. C., J. Pickworth, J. H. Robertson, K. N. Trueblood, R. J. Prosen, and J. G. White. 1955. The crystal structure of the hexacarboxylic acid derived from B_{12} and the molecular structure of the vitamin. *Nature* 176:325-328.

5. Roth, J. R., J. G. Lawrence, and T. A. Bobik. 1996. Cobalamin (coenzyme B12): synthesis and biological significance. *Annu. Rev. Microbiol.* 50:137-181.

6. Pezacka, E., R. Green, and D. W. Jacobsen. 1990. Glutathionylcobalamin as an intermediate in the formation of cobalamin coenzymes. *Biochem. Biophys. Res. Comm.* 169:443-450.

7. Ribichich, K. F., R. C. Georg, and S. L. Gomes. 2006. Comparative EST analysis provides insights into the basal aquatic fungus *Blastocladiella emersonii. BMC Genomics* 7:177

8. Battersby, A. R. and F. J. Leeper. 1999. Biosynthesis of B_{12} in the aerobic organism *Pseudomonas denitrificans*. In Chemistry and Biochemistry of B_{12}. R. Banerjee, editor. John Wiley and Sons, New York. 507-536.

9. Scott, A. I., C. A. Roessner, and P. J. Santander. 1999. B_{12} biosynthesis: the anaerobic pathway. In Chemistry and Biochemistry of B_{12}. R. Banerjee, editor. John Wiley and Sons, New York. 537-556.

10. Riera, J., F. T. Robb, R. Weiss, and M. Fontecave. 1997. Ribonucleotide reductase in the archaeon Pyrococcus furiosus: a critical enzyme in the evolution of DNA genomes? *Proc. Natl. Acad. Sci USA.* 94:475-478.

11. Benner, S. A., A. D. Ellington, and A. Tauer. 1989. Modern metabolism as a palimpsest of the RNA world. *Proc. Natl. Acad. Sci USA.* 86:7054-7058.

12. Hodgson, G. W. and C. Ponnamperuma. 1968. Prebiotic porphyrin genesis: porphyrins from electric discharge in methane, ammonia and water vapor. *Proc. Natl. Acad. Sci USA.* 59:22-28.

13. Eschenmoser, A. 2006. Vitamin B_{12}: experiments concerning the origin of its molecular structure. *(Angew) Chem. Int. Ed. Engl.* 27:5-39.

14. Warren, M. J. 2006. Finding the final pieces of the vitamin B_{12} biosynthetic jigsaw. *Proc. Natl. Acad. Sci. USA* 103:4799-4800.

15. Stroinski, A. 1987. Adenosylcobamide-dependent reactions. In Comprehensive B_{12}. Z. Schneider and A. Stroinski, editors. W. de Gruyter, Berlin. 226-59.

16. Rickes, E. L., N. G. Brink, F. R. Koniuszy, T. R. Wood, and K. Folkers. 1948. Vitamin B_{12}, a cobalt complex. *Science* 108:134-135.

17. Smith, E. L. and L. F. Parker. 1948. Purification of anti-pernicious anaemia factor. *Proc. Biochem. Soc.* 43:viii.

18. Ellis, B., V. Petrow, and G. F. Snook. 1949. The chemistry of anti-pernicious anaemia factors; the ninhydrin-reacting hydrolytic fragment of vitamin B12. *J Pharm Pharmacol* 12:950-956.

19. Flavin, M., P. J. Ortiz, and S. Ochoa. 1955. Metabolism of propionic acid in animal tissues. *Nature* 176:823-6.

20. Weissbach, H., J. Toohey, and H. A. Barker. 1959. Isolation and properties of B_{12} coenzymes containing benzimidazole or dimethylbenzimidazole. *Proc. Natl. Acad. Sci USA.* 45:521-5.

21. Barker, H. A., H. Weissbach, and R. D. Smyth. 1958. A coenzyme containing pseudovitamin B_{12}. *Proc. Natl. Acad. Sci. USA* 44:1093-1097.

22. Smith, R. M. and K. J. Monty. 1959. Vitamin B_{12} and propionate metabolism. *Biochem. Biophys. Res. Comm.* 1:105-109.

23. Gurnani, S., S. P. Mistry, and B. C. Johnson. 1960. Function of vitamin B_{12} in methylmalonate metabolism. I. Effect of a cofactor form of B_{12} on the activity of methylmalonyl-CoA isomerase. *Biochim. Biophys. Acta* 38:187-188.

24. Stern, J. R. and D. C. Friedmann. 1960. Vitamin B_{12} and methylmalonyl-CoA isomerase. I. Vitamin B_{12} and propionate metabolism. *Biochem. Biophys. Res. Comm.* 2:82

25. Guest, J. R., C. W. Helleiner, M. J. Cross, and D. D. Woods. 1960. Cobalamin and the synthesis of methionine by ultrasonic extracts of *Escherichia coli*. *Biochem. J.* 76:396-412.

26. Taylor, R. T. and H. Weissbach. 1967. N^5-methyltetrahydrofolate-homocysteine transmethylase. Partial purification and properties. *J. Biol. Chem.* 242:1502-1508.

27. Taylor, R. T. and H. Weissbach. 1968. Escherichia coli B N^5-methyltetrahydrofolate-homocysteine vitamin B_{12} transmethylase: formation and photolability of a methylcobalamin enzyme. *Arch. Biochem. Biophys.* 123:109-126.

28. Toroya, T. and K. Mori. 1999. A reactivating factor for coenzyme B_{12}-dependent diol dehydratase. *J. Biol. Chem.* 274:3372-3377.

29. Matthews, R. G. and M. L. Ludwig. 2001. Microbial modeling of human disease: homocysteine metabolism. In Homocysteine in Health and Disease. R. Carmel and D. W. Jacobsen, editors. Cambridge University Press, Cambridge. 100-112.

30. Drennan, C. L., S. Huang, J. T. Drummond, R. G. Matthews, and M. L. Ludwig. 1994. How a protein binds B_{12}: a 3.0 Å X-ray structure of B_{12}-binding domains of methionine synthase. *Science* 266:1669-1674.

31. Garrow, T. A. 2001. Betaine-dependent remethylation. In Homocysteine in Health and Disease. R. Carmel and D. W. Jacobsen, editors. Cambridge University Press, Cambridge. 145-152.

32. Clarke, S. and K. Banfield. 2001. S-adenosylmethionine-dependent methyltransferases. In Homocysteine in Health and Disease. R. Carmel and D. W. Jacobsen, editors. Cambridge University Press, New York. 63-78.

33. de la Haba, G. and G. L. Cantoni. 1959. The enzymatic synthesis of S-adenosyl-L-homocysteine from adenosine and homocysteine. *J. Biol. Chem.* 234:603-608.

34. Chanarin, I., R. Deacon, M. Lumb, M. Muir, and J. Perry. 1985. Cobalamin-folate interactions: a critical review. *Blood* 66:479-489.

35. Rosenblatt, D. S., B. A. Cooper, S. Lue-Shing, P. W. K. Wong, S. Berlow, K. Narisawa, and R. Baumgartner. 1979. Folate distribution in cultured human cells. Studies on 5,10-CH_2-H_4PteGlu reductase deficiency. *J. Clin. Invest.* 63:1019-1025.

36. Noronha, J. M. and M. Silverman. 1962. Distribution of folic acid derivatives in natural material. I. Chicken liver folates. *J. Biol. Chem.* 237:3299-3302.

37. Stabler, S. P. and R. H. Allen. 2004. Vitamin B_{12} deficiency as a worldwide problem. *Ann. Rev. Nutr.* 24:299-326.

38. Banerjee, D. K. and J. B. Chatterjea. 1953. Vitamin B_{12} content of some articles of Indian diets and the effect of cooking on it. *Br. J. Nutr.* 17:385-389.

39. Roberts, R. D. and J. K. G. Webb. 1973. Vitamin B_{12} deficiency in Indian infants. *Br. Med. J.* 3:67

40. Prasannan, P., S. Pike, K. Peng, B. Shane, and D. R. Appling. 2003. Human mitochondrial C_1-tetrahydrofolate synthase. Gene structure, tissue distribution of the mRNA, and immunolocalization in Chinese hamster ovary cells. *J. Biol. Chem.* 278:43178-43187.

41. Chanarin, I. and E. Stephenson. 1988. Vegetarian diet and cobalamin deficiency: their association with tuberculosis. *J. Clin. Pathol.* 41:759-762.

42. Rosenberg, L. E., A.-C. Lilljeqvist, and Y. E. Hsia. 1968. Methylmalonic aciduria: An inborn error leading to metabolic acidosis, long-chain ketonuria and intermittent hyperglycinemia. *N. Engl. J. Med.* 278:1319

43. Lindblad, B., P. Olin, B. Svanberg, and R. Zetterstrom. 1968. Methylmalonic acidemia. *Acta. Paediatr. Scand.* 57:417

44. Lindblad, B., K. Lindstrand, B. Svanberg, and R. Zetterstrom. 1969. The effect of cobamide coenzyme in methylmalonic acidemia. *Acta. Paediatr. Scand.* 58:178

45. Oberholzer, V. C., B. Levin, E. A. Burgess, and W. F. Young. 1967. Methylmalonic aciduria: An inborn error of metabolism leading to chronic metabolic acidosis. *Arch. Dis. Child.* 42:492

46. Stokke, O., L. Eldjarn, K. R. Norum, J. Steen-Johnsen, and S. Halvorsen. 1967. Methylmalonic aciduria: A new inborn error of metabolism which may cause fatal acidosis in the neonatal period. *Scand. J. Clin. Lab. Invest.* 20:313-318.

47. Morrow, G., L. A. Barness, G. J. Cardinale, R. H. Abeles, and J. G. Flaks. 1969. Congenital methylmalonic acidemia: enzymatic evidence for two forms of the disease. *Proc. Natl. Acad. Sci. USA* 63:191-197.

48. Mudd, S. H., H. L. Levy, and R. H. Abeles. 1969. Derangement in B_{12} metabolism leading to homocystinemia, cystathioninemia and methylmalonic aciduria. *Biochem. Biophys. Res. Comm.* 35:121-125.

49. Goodman, S. I., P. G. Moe, and K. B. Hammond. 1970. Homocystinuria with methylmalonic aciduria: two cases in a sibship. *Biochem. Med.* 4:500-515.

50. Schuh, S., D. S. Rosenblatt, B. A. Cooper, M.-L. Schroeder, A. J. Bishop, L. E. Seargeant, and J. C. Haworth. 1984. Homocystinuria and megaloblastic anemia responsive to vitamin B_{12} therapy. An inborn error of metabolism due to a defect in cobalamin metabolism. *N. Engl. J. Med.* 310:686-690.

51. Rosenblatt, D. S., B. A. Cooper, A. Pottier, H. Lue-Shing, N. Matiaszuk, and K. Grauer. 1984. Altered vitamin B_{12} 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.

52. Watkins, D. and D. S. Rosenblatt. 1988. Genetic heterogeneity among patients with methylcobalamin deficiency. Definition of two complementation groups, cblE and cblG. *J. Clin. Invest.* 81:1690-1694.

53. Watkins, D. and D. S. Rosenblatt. 1989. Functional methionine synthase deficiency (cblE and cblG): clinical and biochemical heterogeneity. *Am. J. Med. Genet.* 34:427-434.

54. Mahoney, M. J. and L. E. Rosenberg. 1971. Synthesis of cobalamin coenzymes by human cells in culture. *J. Lab. Clin. Med.* 78:302-308.

55. Mahoney, M. J., A. C. Hart, V. D. Steen, and L. E. Rosenberg. 1975. Methylmalonicacidemia: biochemical heterogeneity in defects of 5'-deoxyadenosylcobalamin synthesis. *Proc. Nat. Acad. Sci. USA* 72:2799-2803.

56. Gravel, R. A., M. J. Mahoney, F. H. Ruddle, and L. E. Rosenberg. 1975. Genetic complementation in heterokaryons of human fibroblasts defective in cobalamin metabolism. *Proc. Natl. Acad. Sci. USA.* 72:3181-3185.

57. Fenton, W. A., A. M. Hack, J. P. Kraus, and L. E. Rosenberg. 1987. Immunochemical studies of fibroblasts from patients with methylmalonyl-CoA mutase apoenzyme deficiency: detection of a mutation interfering with mitochondrial import. *Proc. Natl. Acad. Sci. USA* 84:1421-1424.

58. Willard, H. F. and L. E. Rosenberg. 1980. Inherited methylmalonyl CoA mutase apoenzyme deficiency in human fibroblasts. Evidence for allelic heterogeneity, genetic compounds, and codominant expression. *J. Clin. Invest.* 65:690-698.

59. Willard, H. F., I. S. Mellman, and L. E. Rosenberg. 1978. Genetic complementation among inherited deficiencies of methylmalonyl-CoA mutase activity: evidence for a new class of human cobalamin mutant. *Am. J. Hum. Genet.* 30:1-13.

60. Watkins, D. and D. S. Rosenblatt. 1986. Failure of lysosomal release of vitamin B_{12}: a new complementation group causing methylmalonic aciduria. *Am. J. Hum. Genet.* 39:404-408.

61. Rosenblatt, D. S., A. Hosack, N. V. Matiaszuk, B. A. Cooper, and R. Laframboise. 1985. Defect in vitamin B_{12} release from lysosomes: newly described inborn error of vitamin B_{12} metabolism. *Science* 228:1319-1321.

62. Laframboise, R., B. A. Cooper, and D. S. Rosenblatt. 1992. Malabsorption of vitamin B_{12} from the intestine in a child with *cblF* disease: evidence for lysosomal-mediated absorption. *Blood* 80:291-292.

63. Rosenblatt, D. S., R. Laframboise, J. Pichette, P. Langevin, B. A. Cooper, and T. Costa. 1986. New disorder of vitamin B_{12} metabolism (cobalamin F) presenting as methylmalonic aciduria. *Pediatrics* 78:51-54.

64. Watkins, D. and D. S. Rosenblatt. 1986. Heterogeneity in functional methionine synthase deficiency. In Chemistry and Biology of Pteridines. B. A. Cooper and V. M. Whitehead, editors. Walter de Gruyter & Co. Berlin. 713-716.

65. Watkins, D., N. Matiaszuk, and D. S. Rosenblatt. 2000. Complementation studies in the *cblA* class of inborn error of cobalamin metabolism: evidence for interallelic complementation and for a new complementation class (*cblH*). *J. Med. Genet.* 37:510-513.

66. Suormala, T., M. R. Baumgartner, D. Coelho, P. Zavadakova, V. Kozich, H. G. Koch, M. Berghauser, J. E. Wraith, A. Burlina, A. Sewell, J. Herwig, and B. Fowler. 2004. The cblD defect causes either isolated or combined deficiency of methylcobalamin and adenosylcobalamin synthesis. *J. Biol. Chem.* 279:42742-42749.

67. Platica, O., R. Janeczko, E. V. Quadros, A. Regec, R. Romain, and S. P. Rothenberg. 1991. The cDNA sequence and the deduced amino acid sequence of human transcobalamin II show homology with rat intrinsic factor and human transcobalamin I. *J. Biol. Chem.* 266:7860-7863.

68. Li, N., S. Seetharam, and B. Seetharam. 1995. Genomic structure of human transcobalamin II: comparison to human intrinsic factor and transcobalamin I. *Biochem. Biophys. Res. Comm.* 208:756-764.

69. Seetharam, B. and R. R. Yammani. 2003. Cobalamin transport proteins and their cell-surface receptors. *Expert Rev. Molec. Med.* 5:1-18.

70. Hewitt, J. E., M. M. Gordon, R. T. Taggart, T. K. Mohandas, and D. H. Alpers. 1991. Human intrinsic factor: characterization of cDNA and genomic clones and localization to human chromosome 11. *Genomics* 10:432-440.

71. Allen, N. C., B. Seetharam, E. R. Podell, and D. H. Alpers. 1978. Effect of proteolytic enzymes on the binding of cobalamin to R protein and intrinsic factor. In vitro evidence that a failure to partially degrade protein is responsible for cobalamin malabsorption in pancreatic insufficiency. *J. Clin. Invest.* 61:47-54.

72. Allen, R. H., B. Seetharam, N. C. Allen, E. R. Podell, and D. H. Alpers. 1978. Correction of cobalamin malabsorption in pancreatic insufficiency with a cobalamin analogue that binds with high affinity to R protein but not to intrinsic factor. In vivo evidence that a failure to partially degrade R protein is responsible for cobalamin malabsorption in pancreatic insufficiency. *J. Clin. Invest.* 62:1628-1634.

73. Yang-Feng, T. L., N. Berliner, P. Deverajan, and J. Johnston. 1991. Assignment of two human neutrophil secondary protein granule protein genes, transcobalamin I and neutrophil collagenase, to chromosome 11. *Cytogenet. Cell Genet.* 58:1974(Abstr.)

74. Fernandes-Costa, F. and J. Metz. 1982. Vitamin B_{12} binders (transcobalamins) in serum. *Crit. Rev. Clin. Lab. Sci.* 18:1-30.

75. Ogawa, K., H. Kudo, Y. C. Kim, Y. Nakashima, G. Ohshio, and H. Yamabe. 1988. Expression of vitamin B_{12} R-binder in breast tumors. An immunohistochemical study. *Arch. Pathol. Lab. Med.* 112:1117-1120.

76. Lee, E. Y., B. Seetharam, D. H. Alpers, and K. DeSchryver-Kecskemeti. 1989. Immunohistochemical survey of cobalamin-binding proteins. *Gastroenterology* 97:1171-1180.

77. Moestrup, S. K., R. Kozyraki, M. Kristiansen, J. H. Kaysen, H. H. Rasmussen, D. Brault, F. Pontillon, F. O. Goda, E. I. Christensen, T. G. Hammond, and P. J. Verroust. 1998. The intrinsic factor-vitamin B_{12} receptor and target of teratogenic antibodies is a megalin-binding peripheral protein with homology to developmental proteins. *J. Biol. Chem.* 273:5235-5242.

78. Aminoff, M., J. E. Carter, R. B. Chadwick, C. Johnson, R. Gräsbeck, M. A. Abdelaal, H. Broch, L. B. Jenner, P. J. Verroust, S. Moestrup, A. de la Chapelle, and R. Krahe. 1999. Mutations in CUBN, encoding the intrinsic factor-vitamin B_{12} receptor, cubilin, cause hereditary megaloblastic anaemia 1. *Nature Genet.* 21:309-313.

79. Tanner, S. M., M. Aminoff, F. A. Wright, S. Liyanarachchi, M. Kuronen, A. Saarinen, O. Massika, H. Mandel, H. Broch, and A. de la Chapelle. 2003. Amnionless, essential for mouse gastrulation, is mutated in recessive hereditary megaloblastic anemia. *Nature Genetics* 33:426-429.

80. He, Q., H. Madsen, A. Kilkenney, B. Gregory, E. I. Christensen, H. Vorum, P. Hojrup, A. A. Schäffer, E. F. Kirkness, S. M. Tanner, A. de la Chapelle, U. Giger, S. K. Moestrup, and J. C. Fyfe. 2005. Amnionless function is required for cubilin brush-border expression and intrinsic factor-cobalamin (vitamin B_{12}) absorption in vivo. *Blood* 106:1447-1453.

81. Acquaviva, C., J.-F. Benoist, I. Callebaut, N. Guffon, H. O. de Baulny, A. Touati, D. Porquet, and J. Elion. 2001. N219Y, a new frequent mutation among *mut*^{0} forms of methylmalonic acidemia in Caucasian patients. *Eur. J. Hum. Genet.* 9:577-582.

82. Sennett, C., L. E. Rosenberg, and I. S. Mellman. 1981. Transmembrane transport of cobalamin in prokaryotic and eukaryotic cells. *Ann. Rev. Biochem.* 50:1053-1086.

83. Quadros, E. V., S. P. Rothenberg, Y.-C. E. Pan, and S. Stein. 1986. Purification and molecular characterization of human transcobalamin II. *J. Biol. Chem.* 261:15455-15460.

84. Pletsch, Q. A. and J. W. Coffey. 1971. Intracellular distribution of radioactive vitamin B_{12} in rat liver. *J. Biol. Chem.* 246:4619-4629.

85. Wuerges, J., G. Garau, S. Geremia, S. N. Fedosov, and T. E. Petersen. 2006. Structural basis for mammalian vitamin B_{12} transport by transcobalamin. *Proc. Natl. Acad. Sci. USA* 103:4386-4391.

86. Fedosov, S. N., N. U. Fedosova, L. Berglund, S. Moestrup, E. Nexo, and T. E. Petersen. 2005. Composite organization of the cobalamin binding and cubilin recognition sites of intrinsic factor. *Biochemistry* 44:3604-3614.

87. Fedosov, S. N., N. U. Fedosova, L. Berglund, S. K. Moestrup, E. Nexo, and T. E. Petersen. 2004. Assembly of the intrinsic factor domains and oligomerization of the protein in the presence of cobalamin. *Biochemistry* 43:15095-15102.

88. Ramanujam, K. S., S. Seetharam, M. Ramasamy, and B. Seetharam. 1991. Expression of cobalamin transport proteins and cobalamin transcytosis by colon adenocarcinoma cells. *Am. J. Physiol.* 260:G416-G422.

89. Robertson, J. A. and N. D. Gallagher. 1985. Intrinsic factor-cobalamin accumulates in the ilea of mice treated with chloroquine. *Gastroenterology* 89:1353-1359.

90. Robertson, J. A. and N. D. Gallagher. 1985. In vivo evidence that cobalamin is absorbed by receptor-mediated endocytosis in the mouse. *Gastroenterology* 88:908-912.

91. Rothenberg, S. P., J. P. Weiss, and R. Cotter. 1978. Formation of transcobalamin II-vitamin B_{12} complex by guinea-pig ileal mucosa in organ culture after *in vitro* incubation with intrinsic factor-vitamin B_{12}. *Br. J. Haematol.* 40:401-414.

92. Chanarin, I., M. Muir, A. Hughes, and A. V. Hoffbrand. 1978. Evidence for intestinal origin of transcobalamin-II during vitamin-B_{12} absorption. *Br. Med. J.* 1:1453-1455.

93. Kapadia, C. R., D. E. Schafer, R. M. Donaldson, and E. R. Ebersole. 1979. Evidence for involvement of cyclic nucleotides in intrinsic factor secretion by isolated rabbit gastric mucosa. *J. Clin. Invest.* 64:1044-1049.

94. Kolhouse, J. F., H. Kondo, N. C. Allen, E. Podell, and R. H. Allen. 1978. Cobalamin analogues are present in human plasma and can mask cobalamin deficiency because current isotope dilution assays are not specific for true cobalamin. *N. Engl. J. Med.* 299:785-792.

95. Cooper, B. A. and W. Paranchych. 1961. Selective uptake of specifically bound cobalt-58 vitamin B_{12} by human and mouse tumour cells. *Nature* 191:393-395.

96. Kishimoto, T., M. Tavassoli, R. Green, and D. W. Jacobsen. 1987. Receptors for transferrin and transcobalamin II display segregated distribution on microvilli of leukemia L1210 cells. *Biochem. Biophys. Res. Comm.* 146:1102-1108.

97. Seligman, P. A. and R. H. Allen. 1978. Characterization of the receptor for transcobalamin II isolated from human placenta. *J. Biol. Chem.* 253:1766-1772.

98. Quadros, E. V., P. Sai, and S. P. Rothenberg. 1994. Characterization of the human placental membrane receptor for transcobalamin II-cobalamin. *Arch. Biochem. Biophys.* 308:192-199.

99. Bose, S., S. Seetharam, and B. Seetharam. 1995. Membrane expression and interactions of human transcobalamin II receptor. *J. Biol. Chem.* 270:8152-8157.

100. Quadros, E. V., Y. Nakayama, and J. M. Seqeira. 2005. The binding properties of the human receptor for the cellular uptake of vitamin B_{12}. *Biochem. Biophys. Res. Comm.* 327:1006-1010.

101. DiGirolamo, P. M. and F. M. Huennekens. 1975. Transport of vitamin B_{12} into mouse leukemia cells. *Arch. Biochem. Biophys.* 168:386-393.

102. Youngdahl-Turner, P., I. S. Mellman, R. H. Allen, and L. E. Rosenberg. 1979. Protein mediated vitamin uptake. Adsorptive endocytosis of the transcobalamin II-cobalamin complex by cultured human fibroblasts. *Exp. Cell Res.* 118:127-134.

103. Youngdahl-Turner, P., L. E. Rosenberg, and R. H. Allen. 1978. Binding and uptake of transcobalamin II by human fibroblasts. *J. Clin. Invest.* 61:133-141.

104. Rosenblatt, D., A. Hosack, and N. Matiaszuk. 1987. Expression of transcobalamin II by amniocytes. *Prenat. Diag.* 7:35-39.

105. Chanarin, I. 1979. The Megaloblastic Anaemias. Blackwell Scientific Publications, London.

106. Vassiliadis, A., D. S. Rosenblatt, B. A. Cooper, and J. J. M. Bergeron. 1991. Lysosomal cobalamin accumulation in fibroblasts from a patient with an inborn error of

cobalamin metabolism (cblF complementation group): visualization by electron microscope radioautography. *Exp. Cell Res.* 195:295-302.

107. Fenton, W. A., L. M. Ambani, and L. E. Rosenberg. 1976. Uptake of hydroxocobalamin by rat liver mitochondria. Binding to a mitochondrial protein. *J. Biol. Chem.* 251:6616-6623.

108. Pezacka, E. H. and D. S. Rosenblatt. 1994. Intracellular metabolism of cobalamin. Altered activities of β-axial-ligand transferase and microsomal cob(III)alamin reductase in *cblC* and *cblD* fibroblasts. In Advances in Thomas Adddison's Diseases. H. R. Bhatt, V. H. T. James, G. M. Besser, G. F. Bottazzo, and H. Keen, editors. Journal of Endocrinology, Bristol. 315-323.

109. Mellman, I., H. F. Willard, P. Youngdahl-Turner, and L. E. Rosenberg. 1979. Cobalamin coenzyme synthesis in normal and mutant fibroblasts. Evidence for a processing enzyme activity deficient in *cblC* cells. *J. Biol. Chem.* 254:11847-11853.

110. Leclerc, D., E. Campeau, P. Goyette, C. E. Adjalla, B. Christensen, M. Ross, P. Eydoux, D. S. Rosenblatt, R. Rozen, and R. A. Gravel. 1996. Human methionine synthase: cDNA cloning and identification of mutations in patients of the *cblG* complementation group of folate/cobalamin disorders. *Hum. Molec. Genet.* 5:1867-1874.

111. Li, Y. N., S. Gulati, P. J. Baker, L. C. Brody, R. Banerjee, and W. D. Kruger. 1996. Cloning, mapping and RNA analysis of the human methionine synthase gene. *Hum. Molec. Genet.* 5:1851-1858.

112. Chen, L. H., M.-L. Liu, H.-Y. Hwang, L.-S. Chen, J. Korenberg, and B. Shane. 1997. Human methionine synthase. cDNA cloning, gene localization, and expression. *J. Biol. Chem.* 272:3628-3634.

113. Gulati, S., P. Baker, Y. N. Li, B. Fowler, W. Kruger, L. C. Brody, and R. Banerjee. 1996. Defects in human methionine synthase in cblG patients. *Hum. Molec. Genet.* 5:1859-1865.

114. Dixon, M. M., S. Huang, R. G. Matthews, and M. L. Ludwig. 1996. The structure of the C-terminal domain of methionine synthase: presenting S-adenosylmethionine for reductive methylation of B_{12}. *Structure* 4:1263-1275.

115. Evans, J. C., D. P. Huddler, M. T. Hilgers, G. Romanchuk, R. G. Matthews, and M. L. Ludwig. 2004. Structures of the N-terminal modules imply large domain motions during catalysis by methionine synthase. *Proc. Natl. Acad. Sci. USA* 101:3729-3736.

116. Taylor, R. T. 1982. B_{12}-dependent methionine biosynthesis. In B_{12}. D. Dolphin, editor. John Wiley & Sons, New York. 307-355.

117. Matthews, R. G. 1984. Methionine biosynthesis. In Folates and Pterins. Volume 1. Chemistry and Biochemistry of Folates. R. L. Blakley and S. J. Benkovic, editors. John Wiley & Sons, New York. 497-553.

118. Utley, C. S., P. D. Marcell, R. H. Allen, A. C. Antony, and J. F. Kolhouse. 1985. Isolation and characterization of methionine synthetase from human placenta. *J. Biol. Chem.* 260:13656-13665.

119. Ludwig, M. L., C. L. Drennan, and R. G. Matthews. 1996. The reactivity of B_{12} cofactors: the proteins make a difference. *Structure* 4:505-512.

120. Gulati, S., Z. Chen, L. C. Brody, D. S. Rosenblatt, and R. Banerjee. 1997. Defects in auxiliary redox proteins lead to functional methionine synthase deficiency. *J. Biol. Chem.* 272:19171-19175.

121. Banerjee, R. 1997. The yin-yang of cobalamin biochemistry. *Chem. Biol.* 4:175-186.

122. Leclerc, D., A. Wilson, R. Dumas, C. Gafuik, D. Song, D. Watkins, H. H. Q. Heng, J. M. Rommens, S. W. Scherer, D. S. Rosenblatt, and R. A. Gravel. 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.

123. Hall, C. A., R. H. Lindenbaum, E. Arenson, J. A. Begley, and R. C. Chu. 1989. The nature of the defect in cobalamin G mutation. *Clin. Invest. Med.* 12:262-269.

124. Sillaots, S. and D. S. Rosenblatt. 1992. Lysosomal transport of other metabolites. Lysosomal cobalamin transport. In Pathophysiology of Lysosomal transport. J. G. Thoene, editor. CRC Press, Ann Arbor. 201-230.

125. Wilson, A., D. Leclerc, F. Saberi, E. Campeau, H. Y. Hwang, B. Shane, J. A. Phillips, D. S. Rosenblatt, and R. A. Gravel. 1998. Functionally null mutations in patients with the *cblG*-variant form of methionine synthase deficiency. *Am. J. Hum. Genet.* 63:409-414.

126. Ludwig, M. L. and R. G. Matthews. 1997. Structure-based perspectives on B_{12}-dependent enzymes. *Ann. Rev. Biochem.* 66:269-313.

127. Fujii, K. and F. M. Huennekens. 1974. Activation of methionine synthase by a reduced triphosphopyridine nucleotide-dependent flavoprotein system. *J. Biol. Chem.* 249:6745-6753.

128. Katz, J. and I. L. Chaikoff. 1955. Synthesis via the Krebs' cycle in the utilization of acetate by rat liver slices. *Biochim. Biophys. Acta.* 1:87-101.

129. Harding, C. O., G. Arnold, L. A. Barness, J. A. Wolff, and D. S. Rosenblatt. 1997. Functional methionine synthase deficiency due to cblG disorder: a report of two patients and a review. *Am. J. Med. Genet.* 71:384-390.

130. Kolhouse, J. F., C. Utley, and R. H. Allen. 1980. Isolation and characterization of methylmalonyl-CoA mutase from human placenta. *J. Biol. Chem.* 255:2708-2712.

131. Fenton, W. A., A. M. Hack, H. F. Willard, A. Gertler, and L. E. Rosenberg. 1982. Purification and properties of methylmalonyl coenzyme A mutase from human liver. *Arch. Biochem. Biophys.* 214:815-823.

132. Janata, J., N. Kogekar, and W. A. Fenton. 1997. Expression and kinetic characterization of methylmalonyl-CoA mutase from patients with the mut$^-$ phenoype: Evidence for naturally occurring interallelic complementation. *Hum. Mol. Genet.* 6:1457

133. Willard, H. F. and L. E. Rosenberg. 1980. Interactions of methylmalonyl CoA mutase from normal human fibroblasts with adenosylcobalamin and methylmalonyl CoA: evidence for non-equivalent active sites. *Arch. Biochem. Biophys.* 200:130-139.

134. Halpern, J. 1985. Mechanisms of coenzyme B_{12}-dependent rearrangements. *Science* 227:869-875.

135. Padmakumar, R. and R. Banerjee. 1997. Evidence that cobalt-carbon bond homolysis is coupled to hydrogen atom abstraction from substrate in methylmalonyl-CoA mutase. *Biochemistry* 36:3713-3718.

136. Kellermeyer, R. W. and H. G. Wood. 1962. Methylmalonyl isomerase: a study of the mechanism of isomerization. *Biochemistry* 1:1124-1131.

137. Phares, E. F., M. V. Long, and S. F. Carson. 1962. An intramolecular rearrangement in the methylmalonyl isomerase reaction as demonstrated by positive and negative ion mass analysis of succinic acid. *Biochem. Biophys. Res. Comm.* 8:142-146.

138. Francalanci, F., N. K. Davis, J. Q. Fuller, D. Murfitt, and P. F. Leadlay. 1986. The subunit structure of methylmalonyl-CoA mutase from *Propionibacterium shermanii. Biochem. J.* 236:489-494.

139. Marsh, E. N. and P. F. Leadlay. 1989. Methylmalonyl-CoA mutase from *Propionibacterium shermanii.* Evidence for the presence of two masked cysteine residues. *Biochem. J.* 260:339-343.

140. Thoma, N. H. and P. F. Leadlay. 1998. Mechanistic and structural studies on methylmalonyl-CoA mutase. *Biochem. Soc. Trans.* 3:293-298.

141. Mancia, F., N. H. Keep, A. Nakagawa, P. F. Leadlay, S. McSweeney, B. Rasmussen, P. Bösecke, O. Diat, and P. R. Evans. 1996. How coenzyme B_{12} radicals are generated:

the crystal structure of methylmalonyl-coenzyme A mutase at 2 Å resolution. *Structure* 4:339-350.

142. Thoma, N. H., T. W. Meier, P. R. Evans, and P. F. Leadlay. 1998. Stabilization of radical intermediates by an active-site tyrosine residue in methylmalonyl-CoA mutase. *Biochemistry* 37:14386-14393.

143. Drennan, C. L., R. G. Matthews, and M. L. Ludwig. 1994. Cobalamin-dependent methionine synthase: the structure of a methylcobalamin-binding fragment and implications for other B_{12}-dependent enzymes. *Curr. Opin. Struct. Biol.* 4:919-929.

144. Mancia, F. and P. R. Evans. 1998. Conformational changes on substrate binding to methylmalonyl CoA mutase and new insights into the free radical mechanism. *Structure* 6:711-720.

145. Ledley, F. D., M. R. Lumetta, H. Y. Zoghbi, P. Van Tuinen, S. A. Ledbetter, and D. H. Ledbetter. 1988. Mapping of human methylmalonyl CoA mutase (MUT) locus on chromosome 6. *Am. J. Hum. Genet.* 42:839-846.

146. Jansen, R., F. Kalousek, W. A. Fenton, L. E. Rosenberg, and F. D. Ledley. 1989. Cloning of full-length methylmalonyl-CoA mutase from a cDNA library using the polymerase chain reaction. *Genomics* 4:198-205.

147. Nham, S.-U., M. F. Wilkemeyer, and F. D. Ledley. 1990. Structure of the human methylmalonyl-CoA mutase (MUT) locus. *Genomics* 8:710-716.

148. Wilkemeyer, M. F., A. M. Crane, and F. D. Ledley. 1990. Primary structure and activity of mouse methylmalonyl-CoA mutase. *Biochem. J.* 271:449-455.

149. Kolhouse, J. F., C. Utley, W. A. Fenton, and L. E. Rosenberg. 1981. Immunochemical studies on cultured fibroblasts from patients with inherited methylmalonic acidemia. *Proc. Natl. Acad. USA* 78:7737-7741.

150. Willard, H. F. and L. E. Rosenberg. 1977. Inherited deficiencies of human methylmalonyl CoA mutase activity: reduced affinity of mutant apoenzyme for adenosylcobalamin. *Biochem. Biophys. Res. Comm.* 78:927-934.

151. Morrow, G., B. Revsin, R. Clark, J. Lebowitz, and D. T. Whelan. 1978. A new variant of methylmalonic acidemia - defective coenzyme-apoenzyme binding in cultured fibroblasts. *Clin. Chim. Acta* 85:67-72.

152. Dobson, C. M., T. Wai, D. Leclerc, A. Wilson, X. Wu, C. Doré, T. J. Hudson, D. S. Rosenblatt, and R. A. Gravel. 2002. Identification of the gene responsible for the *cblA* complementation group of vitamin B_{12}-responsive methylmalonic acidemia based on analysis of prokaryotic gene arrangements. *Proc. Nat. Acad. Sci. USA* 99:15554-15559.

153. Dobson, C. M., T. Wai, D. Leclerc, H. Kadir, M. Narang, J. P. Lerner-Ellis, T. J. Hudson, D. S. Rosenblatt, and R. A. Gravel. 2002. Identification of the gene responsible for the *cblB* complementation group of vitamin B_{12}-dependent methylmalonic aciduria. *Hum. Molec. Genet.* 26:3361-3369.

154. Bobik, T. A. and M. E. Rasche. 2001. Identification of the human methylmalonyl-CoA racemase gene based on the analysis of prokaryotic gene arrangements. Implications for decoding the human genome. *J. Biol. Chem.* 276:37194-37198.

155. Dobson, C. M., A. Gradinger, N. Longo, X. Wu, D. Leclerc, J. Lerner-Ellis, M. Lemieux, C. Belair, D. Watkins, D. S. Rosenblatt, and R. A. Gravel. 2006. Homozygous nonsense mutation in the *MCEE* gene and siRNA suppression of methylmalonyl-CoA epimerase expression: a novel cause of mild methylmalonic aciduria. *Molec. Genet. Metab.* 88:327-333.

156. Bikker, H., H. D. Bakker, N. G. G. M. Abeling, B. T. Poll-The, W. J. Kleijer, D. S. Rosenblatt, H. R. Waterham, R. J. A. Wanders, and M. Duran. 2006. A homozygous nonsense mutation in the methylmalonyl-CoA epimerase gene (MCEE) results in mild methylmalonic aciduria. *Hum. Mutat.* 27:640-643.

157. Kolhouse, J. F. and R. H. Allen. 1977. Recognition of two intracellular cobalamin binding proteins and their identification as methylmalonyl-CoA mutase and methionine synthetase. *Proc. Natl. Acad. Sci. USA* 74:921-925.

158. Watanabe, F. and Y. Nakano. 1997. Purification and characterization of aquacobalamin reductases from mammals. *Methods Enzymol.* 281:295-305.

159. Watanabe, F., H. Saido, R. Yamaji, K. Miyatake, Y. Isegawa, A. Ito, T. Yubisui, D. S. Rosenblatt, and Y. Nakano. 1996. Mitochondrial NADH- or NADPH-linked aquacobalamin reductase activity is low in human skin fibroblasts with defects in synthesis of cobalamin coenzymes. *J. Nutr.* 126:2947-2951.

160. Celis, R. T., P. F. Leadlay, I. Roy, and A. Hansen. 1998. Phosphorylation of the periplasmic binding protein in two transport systems for arginine incorporation in *Escherichia coli* K-12 is unrelated to the function of the transport system. *J. Bacteriol.* 180:4828-4833.

161. Higgins, C. F. and G. F. Ames. 1981. Two periplasmic transport proteins which interact with a common membrane receptor show extensive homology: complete nucleotide sequences. *Proc. Natl. Acad. Sci USA.* 78:6038-6042.

162. Haller, T., T. Buckel, J. Retey, and J. A. Gerlt. 2000. Discovering new enzymes and metabolic pathways: conversion of succinate to propionate by *Escherichia coli*. *Biochemistry* 39:4622-4629.

163. Locher, K. P., A. T. Lee, and D. C. Rees. 2002. The E. coli BtuCD structure: a framework for ABC transporter architecture and mechanism. *Science* 296:1091-1098.

164. Leipe, D. D., E. V. Koonin, and L. Aravind. 2003. Evolution and classification of P-loop kinases and related proteins. *J. Mol. Biol.* 333:781-815.

165. Fenton, W. A. and L. E. Rosenberg. 1978. Mitochondrial metabolism of hydroxocobalamin: synthesis of adenosylcobalamin by intact rat liver mitochondria. *Arch. Biochem. Biophys.* 189:441-447.

166. Chernyak, B. V. and P. Bernardi. 1996. The mitochondrial permeability transition pore is modulated by oxidative agents through both pyridine nucleotides and glutathione at two separate sites. *Eur. J. Biochem.* 238:623-630.

167. Korotkova, N. and M. E. Lidstrom. 2004. MeaB is a component of the methylmalonyl-CoA mutase complex required for protection of the enzyme from inactivation. *J. Biol. Chem.* 279:13652-13658.

168. Enright, A. J., I. Iliopoulos, N. C. Kyrpides, and C. A. Ouzounis. 1999. Protein interaction maps for complete genomes based on gene fusion events. *Nature* 402:86-90.

169. Padovani, D., T. Labunska, and R. Banerjee. 2006. Energetics of interaction between the G-protein chaperone, MeaB, and B_{12}-dependent methylmalonyl-CoA mutase. *J. Biol. Chem.* 281:17838-17844.

170. Banerjee, R. 2006. B12 trafficking in mammals: a case for coenzyme escort service. *ACS Chem. Biol.* 1:149-159.

171. Padovani, D. and R. Banerjee. 2006. Assembly and protection of the radical enzyme, methylmalonyl-CoA mutase, by its chaperone. *Biochemistry* 45:9300-9306.

172. Leal, N. A., S. D. Park, P. E. Kima, and T. A. Bobik. 2003. Identification of the human and bovine ATP:Cob(I)alamin adenosyltransferase cDNAs based on complementation of a bacterial mutant. *J. Biol. Chem.* 278:9227-9234.

173. Zhang, J., C. M. Dobson, X. Wu, J. P. Lerner-Ellis, D. S. Rosenblatt, and R. A. Gravel. 2006. Impact of *cblB* mutations on the function of ATP:cob(I)alamin adenosyltransferase in disorders of vitamin B_{12} metabolism. *Molec. Genet. Metab. 87:315-322.*

174. Fonseca, M. V. and J. C. Escalante-Semerena. 2001. An *in vitro* reducing system for the enzymic conversion of cobalamin to adenosylcobalamin. *J. Biol. Chem.* 276:32101-32108.

175. Bauer, C. B., M. V. Fonseca, H. M. Holden, J. B. Thoden, T. B. Thompson, J. C. Escalante-Semerena, and I. Rayment. 2001. Three-dimensional structure of

ATP:corrinoid adenosyltransferase from Salmonella typhimurium in its free state, complexed with MgATP, or complexed with hydroxycobalamin and MgATP. *Biochemistry* 40:361-374.

176. Johnson, C. L. V., E. Pechonick, S. D. Park, G. D. Havemann, N. A. Leal, and T. A. Bobik. 2001. Functional genomic, biochemical, and genetic characterization of the *Salmonella pduO* gene, an ATP:cob(I)alamin adenosyltransferase gene. *J. Bacteriol.* 183:1577-1584.

177. Kofoid, E., C. Rappleye, I. Stojilkovic, and J. R. Roth. 1999. The 17-gene ethanolamine (eut) operon of Salmonella typhimurium encodes five homologues of carboxysome shell proteins. *J. Bacteriol.* 181:5317-5329.

178. Stich, T. A., M. Yamanishi, R. Banerjee, and T. C. Brunold. 2005. Spectroscopic evidence for the formation of a four-coordinate Co^{2+} cobalamin species upon binding to the human ATP:cobalamin adenosyltransferase. *J. Am. Chem. Soc.* 127:7660-7661.

179. Coulombe, J. T., V. E. Shih, and H. L. Levy. 1981. Massachusetts Metabolic Disorders Screening Program. II. Methylmalonic aciduria. *Pediatrics* 67:26-31.

180. MacMahon, M. 1995. Requesting vitamin B_{12} and folate assays. *Lancet* 346:446-447.

181. Fenton, W. A., R. A. Gravel, and D. S. Rosenblatt. 2001. Disorders of Propionate and Methylmalonate Metabolism. In The Metabolic and Molecular Basis of Inherited Disease. C. R. Scriver, A. L. Beaudet, D. Valle, and W. S. Sly, editors. The McGraw-Hill, New York. 2165-2193.

182. Shapira, S. K., F. D. Ledley, D. S. Rosenblatt, and H. L. Levy. 1991. Ketoacidotic crisis as a presentation of mild ("benign") methylmalonic acidemia. *J. Pediatr.* 119:80-84.

183. Sniderman, L. C., M. Lambert, R. Giguere, C. Auray-Blais, B. Lemieux, R. Labramboise, and D. S. Rosenblatt. 1999. Outcome of individuals with low-moderate methylmalonic aciduria detected through a neonatal screening program. *J. Pediatr.* 134:680

184. Ando, T., K. Tasmussen, W. L. Nyhan, and D. Hull. 1972. 3-Hydroxypropionate: Significance of ß-oxidation of propionate in patients with propionic acidemia and methylmalonic acidemia. *Proc. Natl. Acad. Sci.USA* 69:2807

185. Stokke, O., E. Jellum, L. Eldjarn, and R. Schnitler. 1973. The occurrence of beta-hydroxy-n-valeric acid in a patient with propionic and methylmalonic acidemia. *Clin. Chim. Acta.* 45:391-401.

186. Sakura, N., H. Ono, S. Nomura, H. Ueda, and N. Fujita. 1998. Betaine dose and treatment intervals in therapy for homocystinuria due to 5,10-methylenetetrahydrofolate reductase deficiency. *J. Inherit. Metab. Dis.* 1:84-85.

187. Cooper, B. A. and D. S. Rosenblatt. 1987. Inherited defects of vitamin B_{12} metabolism. *Ann. Rev. Nutr.* 7:291-320.

188. Shevell, M. I. and D. S. Rosenblatt. 1992. The neurology of cobalamin. *Can. J. Neurol. Sci.* 19:472-486.

189. Rosenblatt, D. S., I. T. Thomas, D. Watkins, B. A. Cooper, and R. W. Erbe. 1987. Vitamin B_{12} responsive homocystinuria and megaloblastic anemia: heterogeneity in methylcobalamin deficiency. *Am. J. Med. Genet.* 26:377-383.

190. Carmel, R., D. Watkins, S. I. Goodman, and D. S. Rosenblatt. 1988. Hereditary defect of cobalamin metabolism (*cblG* mutation) presenting as a neurologic disorder in adulthood. *N. Engl. J. Med.* 318:1738-1741.

191. Hall, C. A. 1990. Function of vitamin B_{12} in the central nervous system as revealed by congenital defects. *Am. J. Hematol.* 34:121-127.

192. Rosenblatt, D. S., A. L. Aspler, M. I. Shevell, B. A. Pletcher, W. A. Fenton, and M. R. Seashore. 1997. Clinical heterogeneity and prognosis in combined methylmalonic aciduria and homocystinuria (*cblC*). *J. Inher. Metab. Dis.* 20:528-538.

193. Shih, V., S. M. Axel, J. C. Tewksbury, D. Watkins, B. A. Cooper, and D. S. Rosenblatt. 1989. Defective lysosomal release of vitamin B_{12} (cblF): a hereditary cobalamin metabolic disorder associated with sudden death. *Am. J. Med. Genet.* 33:555-563.

194. MacDonald, M. R., H. E. Wiltse, J. L. Bever, and D. S. Rosenblatt. 1992. Clinical heterogeneity in two patients with cblF disease. *Am. J. Hum. Genet.* 51:A353(Abstr.)

195. Wong, L. T. K., D. S. Rosenblatt, D. A. Applegarth, and A. G. F. Davidson. 1992. Diagnosis and treatment of a child with cblF disease. *J. Clin. Invest.* 15:A111(Abstr.)

196. Haworth, J. C., L. A. Dilling, R. A. H. Surtees, L. E. Seargeant, H. Lue-Shing, B. A. Cooper, and D. S. Rosenblatt. 1993. Symptomatic and asymptomatic methylenetetrahydrofolate reductase deficiency in two adult brothers. *Am. J. Med. Genet.* 45:572-576.

197. Holme, E., B. Kjellman, and E. Ronge. 1989. Betaine for treatment of homocystinuria caused by methylenetetrahydrofolate reductase deficiency. *Arch. Dis. Child.* 64:1061-1064.

198. Kluijtmans, L. A. J., U. Wendel, E. M. B. Stevens, L. P. W. J. van den Heuvel, F. J. M. Trijbels, and H. J. Blom. 1998. Identification of four novel mutations in severe methylenetetrahydrofolate reductase deficiency. *Eur. J. Hum. Genet.* 6:257-265.

199. Thompson, G. N., R. A. Chalmers, J. H. Walter, J. L. Bresson, S. L. Lyonnet, P. J. Reed, and J. M. Saudubray. 1990. The use of metronidazole in management of methylmalonic and propionic acidaemias. *Eur. J. Pediatr.* 149:792

200. Bain, M. D., S. P. Borriello, B. M. Tracey, M. Jones, P. J. Reed, R. A. Chalmers, and T. E. Stacey. 1988. Contribution of gut bacterial metabolism to human metabolic disease. *Lancet* 331:1078-1079.

201. Carmel, R., A. A. Bedros, J. W. Mace, and S. I. Goodman. 1980. Congenital methylmalonic aciduria-homocystinuria with megaloblastic anemia: observations on response to hydroxocobalamin and on the effect of homocysteine and methionine on the deoxyuridine suppression test. *Blood* 55:570-579.

202. Shinnar, S. and H. S. Singer. 1984. Cobalamin C mutation (methylmalonic aciduria and homocystinuria) in adolescence. *N. Engl. J. Med.* 311:451-454.

203. Mitchell, G. A., D. Watkins, S. B. Melançon, D. S. Rosenblatt, G. Geoffroy, J. Orquin, M. B. Homsy, and L. Dallaire. 1986. Clinical heterogeneity in cobalamin C variant of combined homocystinuria and methylmalonic aciduria. *J. Pediatr.* 108:410-415.

204. Rosenblatt, D. S. and B. A. Cooper. 1986. Inherited disorders of vitamin B_{12} metabolism. *Blood Reviews* 1:177-182.

205. Bellini, C., R. Cerone, W. Bonacci, U. Caruso, C. P. Magliano, G. Serra, B. Fowler, and C. Romano. 1992. Biochemical diagnosis and outcome of 2 years treatment in a patient with combined methylmalonic aciduria and homocystinuria. *Eur. J. Pediatr.* 151:818-820.

206. Bartholomew, D. W., M. L. Batshaw, R. H. Allen, C. R. Roe, D. S. Rosenblatt, D. Valle, and C. A. Francomano. 1988. Therapeutic approaches to cobalamin-C methylmalonic acidemia and homocystinuria. *J. Pediatr.* 112:32-39.

207. Rosenblatt, D. S., B. A. Cooper, S. M. Schmutz, W. A. Zaleski, and R. E. Casey. 1985. Prenatal vitamin B_{12} therapy of a fetus with methylcobalamin deficiency (cobalamin E disease). *Lancet* i:1127-1129.

208. Atkinson, J. L. 2003. Genetic mapping of cobalamin C deficiency: Linkage to chromosome 1p32-34. MA. University of Toronto.

209. Atkinson, J. L., A. Paterson, D. Renaud, J. T. R. Clark, B. Wilcken, D. Bulman, and J. M. Rommens. 2002. Genetic mapping of cobalamin C deficiency: putative linkage to 1p. *Am. J. Hum. Genet.* 71:452

210. Broman, K. W., J. C. Murray, V. C. Sheffield, R. L. White, and J. L. Weber. 1998. Comprehensive human genetic maps: individual and sex-specific variation in recombination. *Am. J. Hum. Genet.* 63:861-869.

211. Dib, C., S. Faure, C. Fizames, D. Samson, N. Drouot, A. Vignal, P. Millasseau, S. Marc, J. Hazan, E. Seboun, M. Lathrop, G. Gyapay, J. Morissette, and J. Weissenbach. 1996. A comprehensive genetic map of the human genome based on 5,264 microsatellites. *Nature* 380:152-154.

212. Lathrop, G. M., J. M. Lalouel, C. Julier, and J. Ott. 1984. Strategies for multilocus linkage analysis in humans. *Proc. Natl. Acad. Sci.* 81:3443-3446.

213. Cottingham, R. W. J., R. M. Idury, and A. A. Schaffer. 1993. Faster sequential genetic linkage computations. *Am. J. Hum. Genet.* 53:252-263.

214. Kruglyak, L., M. J. Daly, M. P. Reeve-Daly, and E. S. Lander. 1996. Parametric and nonparametric linkage analysis: a unified multipoint approach. *Am. J. Hum. Genet.* 58:1347-1363.

215. Kruglyak, L. and E. S. Lander. 1998. Faster multipoint linkage analysis using Fourier transforms. *J. Comput. Biol.* 5:1-7.

216. Spielman, R. S., R. E. McGinnis, and W. J. Ewens. 1993. Transmission test for linkage disequilibrium: the insulin gene region and insulin-dependent diabetes mellitus (IDDM). *Am. J. Hum. Genet.* 52:506-516.

217. Lander, E. S. and D. Botstein. 1987. Homozygosity mapping: a way to map human recessive traits with the DNA of inbred children. *Science* 236:1567-1570.

218. Garrod, A. E. 1902. The incidence of alkaptonuria: a study in chemical individuality. *Lancet* 160:1616-1620.

219. Bateson, W. 1902. Mendel's principles of heredity. *Camb. Univ. Press*

220. Morton, N. E. 1955. Sequential tests for the detection of linkage. *Am. J. Hum. Genet.* 7:277-318.

221. Lerner-Ellis, J. P., J. C. Tirone, P. D. Pawelek, C. Doré, J. L. Atkinson, D. Watkins, C. F. Morel, T. M. Fujiwara, E. Moras, A. R. Hosack, G. V. Dunbar, H. Antonicka, V. Forgetta, C. M. Dobson, D. Leclerc, R. A. Gravel, E. A. Shoubridge, J. W. Coulton, P. Lepage, J. M. Rommens, K. Morgan, and D. S. Rosenblatt. 2006. Identification of the gene responsible for methylmalonic aciduria and homocystinuria, cblC type. *Nature Genet.* 38:93-100.

222. Miano, M. G., S. G. Jacobson, A. Carothers, I. Hanson, P. Teague, J. Lovell, A. V. Cideciyan, N. Haider, E. M. Stone, V. C. Sheffield, and A. F. Wright. 2000. Pitfalls in homozygosity mapping. *Am. J. Hum. Genet.* 67:1348-1351.

223. Broman, K. W. and J. L. Weber. 1999. Long homozygous chromosomal segments in reference families from the Centre d'étude du polymorphisme humain. *Am. J. Hum. Genet.* 65:1493-1500.

224. Lerner-Ellis, J. P., C. M. Dobson, T. Wai, D. Watkins, J. C. Tirone, D. Leclerc, C. Doré, P. Lepage, R. A. Gravel, and D. S. Rosenblatt. 2004. Mutations in the *MMAA* gene in patients with the *cblA* disorder of vitamin B_{12} metabolism. *Hum. Mutat.* 24:509-516.

225. Lerner-Ellis, J. P., A. B. Gradinger, D. Watkins, J. C. Tirone, A. Villeneuve, C. M. Dobson, A. Montpetit, P. Lepage, R. A. Gravel, and D. S. Rosenblatt. 2006. Mutation and biochemical analysis of patients belonging to the *cblB* complementation class of vitamin B_{12}-dependent methylmalonic aciduria. *Molec. Genet. Metab.* 87:219-225.

226. Worgan, L. C., K. Niles, J. C. Tirone, A. Hofmann, A. Verner, A. Sammak, T. Kucic, P. Lepage, and D. S. Rosenblatt. 2006. Spectrum of mutations in *mut* methylmalonic acidemia and identification of a common Hispanic mutation and haplotype. *Hum. Mutat.* 27:31-43.

227. Yang, X., O. Sakamoto, Y. Matsubara, S. Kure, Y. Suzuki, Y. Aoki, N. Sakura, M. Takayanagi, K. Inuma, and T. Ohura. 2004. Mutation analysis of the *MMAA* and *MMAB* genes in Japanese patients with vitamin B_{12}-responsive methylmalonic acidemia: identification of a prevalent *MMAA* mutation. *Molec. Genet. Metab.* 82:329-333.

228. Saridakis, V., A. Yakunin, X. Xu, P. Anandakumar, M. Pennycooke, J. Gu, F. Cheung, J. M. Lew, R. Sanishvili, A. Joachimiak, C. H. Arrowsmith, D. Christendat, and A. M. Edwards. 2004. The structural basis for methylmalonic aciduria. The crystal structure of archaeal ATP:cobalamin adenosyltransferase. *J. Biol. Chem.* 279:23646-23653.

229. Smith, S. E., H. C. Kinney, K. J. Swoboda, and H. L. Levy. 2006. Subacute combined degeneration of the spinal cord in cblC disorder despite treatment with B_{12}. *Molec. Genet. Metab.* 88:138-145.

230. Scott, J. M. and D. G. Weir. 1981. The methyl folate trap. A physiological response in man to prevent methyl group deficiency in kwashiorkor (methionine deficiency) and an explanation for folic-acid induced exacerbation of subacute combined degeneration in pernicious anaemia. *Lancet* 318:337-340.

231. Weir, D. G., S. Keating, A. M. Molloy, J. McPartlin, S. Kennedy, D. Rice, and J. M. Scott. 1988. Methylation deficiency causes vitamin B_{12}-associated neuropathy in the pig. *J. Neurochem.* 51:1949-1952.

232. Schimel, A. M. and M. B. Mets. 2006. The natural history of retinal degeneration in association with cobalamin C (cblC) disease. *Ophthalmic Genet.* 27:9-14.

233. Patton, N., S. Beatty, I. C. Lloyd, and J. E. Wraith. 2000. Optic atrophy in association with cobalamin C (cblC) disease. *Ophthalmic Genet.* 21:151-154.

234. Schrauzer, G. N. and E. Deutsch. 1969. Reactions of cobalt(I) supernucleophiles. The alkylation of vitamin B12s cobaloximes(I), and related compounds. *J. Am. Chem. Soc.* 91:3341-3350.

235. Qureshi, A. A. and D. S. Rosenblatt. 1994. Inherited disorders of cobalamin metabolism. *Crit. Rev. Oncol. Hematol.* 17:133-151.

APPENDIX A: List of Publications

Publications:

Morel CF, **Lerner-Ellis JP**, Rosenblatt DS. Combined methylmalonic aciduria and homocystinuria (cblC): Phenotype-genotype correlations and ethnic-specific observations. *Mol. Genet. Metab.* 2006 May;88:315-321.

Dobson CM, Gradinger A, Longo N, Wu X, Leclerc D, **Lerner-Ellis J**, Lemieux M, Belair C, Watkins D, Rosenblatt DS, Gravel RA. Homozygous nonsense mutation in the MCEE gene and siRNA suppression of methylmalonyl-CoA epimerase expression: A novel cause of mild methylmalonic aciduria. *Mol. Genet. Metab.* 2006 May;88:327-333.

Zhang J, Dobson CM, Wu X, **Lerner-Ellis J**, Rosenblatt DS, Gravel RA. Impact of cblB mutations on the function of ATP:cob(I)alamin adenosyltransferase in disorders of vitamin B(12) metabolism. *Mol. Genet. Metab.* 2006 Apr;87(4):315-22.

Lerner-Ellis JP, Gradinger AB, Watkins D, Tirone JC, Villeneuve A, Dobson CM, Montpetit A, Lepage P, Gravel RA, Rosenblatt DS. Mutation and biochemical analysis of patients belonging to the cblB complementation class of vitamin B_{12}-dependent methylmalonic aciduria. *Mol. Genet. Metab.* 2006 Mar;87(3):219-25.

Lerner-Ellis JP, Tirone JC, Pawelek PD, Dore C, Atkinson JL, Watkins D, Morel CF, Fujiwara TM, Moras E, Hosack AR, Dunbar GV, Antonicka H, Forgetta V, Dobson CM, Leclerc D, Gravel RA, Shoubridge EA, Coulton JW, Lepage P, Rommens JM, Morgan K, Rosenblatt DS. Identification of the gene responsible for methylmalonic aciduria and homocystinuria, cblC type. *Nat. Genet.* 2006 Jan;38(1):93-100.

Lerner-Ellis JP, Dobson CM, Wai T, Watkins D, Tirone JC, Leclerc D, Dore C, Lepage P, Gravel RA, Rosenblatt DS. Mutations in the MMAA gene in patients with the cblA disorder of vitamin B12 metabolism. *Hum. Mutat.* 2004 Dec;24(6):509-16.

Dobson CM, Wai T, Leclerc D, Kadir H, Narang M, **Lerner-Ellis JP**, Hudson TJ, Rosenblatt DS, Gravel RA. Identification of the gene responsible for the cblB complementation group of vitamin B12-dependent methylmalonic aciduria. *Hum. Mol. Genet.* 2002 Dec 15;11(26):3361-9.

Presentations (Oral and Poster):

Lerner-Ellis JP, Tirone JC, Pawelek PD, Dore C, Atkinson JL, Watkins D, Morel CF, Fujiwara TM, Moras E, Hosack AR, Dunbar GV, Antonicka H, Forgetta V, Dobson CM, Leclerc D, Gravel RA, Shoubridge EA, Coulton JW, Lepage P, Rommens JM, Morgan K, Rosenblatt DS. Identification of the gene responsible for methylmalonic aciduria and homocystinuria, cblC type. FASEB – 1-Carbon metabolism, Palm Springs, California, USA. Poster. Aug. 2006.

Lerner-Ellis JP, Tirone JC, Pawelek PD, Dore C, Atkinson JL, Watkins D, Morel CF, Fujiwara TM, Moras E, Hosack AR, Dunbar GV, Antonicka H, Forgetta V, Dobson CM, Leclerc D, Gravel RA, Shoubridge EA, Coulton JW, Lepage P, Rommens JM, Morgan K,

Rosenblatt DS. Identification of the gene responsible for methylmalonic aciduria and homocystinuria, cblC type. Réseau de médicine génétique appliqué - Montreal, PQ, Can. Poster. May 2006.

Lerner-Ellis JP, Tirone JC, Pawelek PD, Dore C, Atkinson JL, Watkins D, Morel CF, Fujiwara TM, Moras E, Hosack AR, Dunbar GV, Antonicka H, Forgetta V, Dobson CM, Leclerc D, Gravel RA, Shoubridge EA, Coulton JW, Lepage P, Rommens JM, Morgan K, Rosenblatt DS. Identification of the gene responsible for methylmalonic aciduria and homocystinuria, cblC type. Canadian Genetic Diseases Network - Saint Sauver, PQ, Can. Oral and Poster. Apr. 2006.

Tanpaiboon P, Brooks BP, **Lerner-Ellis JP**, Sloan, J, Braverman N, Zand D, Lichter-Konecki U, Rosenblatt DS, Venditti. Ophthalmologic Findings in Cobalamin C Deficiency: Examination of Genotype/Phenotype/Biochemical Correlations. ACMG San Diego CA. Poster. Mar. 2006.

Morel C, **Lerner-Ellis JP**, Rosenblatt DS. Phenotype-Genotype Correlations in Combined Methylmalonic Aciduria and Homocystinuria (*cblC*). ACMG San Diego CA. Oral. Mar. 2006.

Lerner-Ellis JP, Tirone JC, Pawelek PD, Dore C, Atkinson JL, Watkins D, Morel CF, Fujiwara TM, Moras E, Hosack AR, Dunbar GV, Antonicka H, Forgetta V, Dobson CM, Leclerc D, Gravel RA, Shoubridge EA, Coulton JW, Lepage P, Rommens JM, Morgan K, Rosenblatt DS. *MMACHC* is mutated in patients with the *cblC* complementation group of vitamin B_{12} metabolism. American Society of Human Genetics - Salt Lake City, Utah, USA. Oral. *AJHG* Suppl. Nov;179(60):29 2005.

Lerner-Ellis JP, Gradinger AB, Watkins D, Tirone JC, Villeneuve A, Dobson CM, Montpetit A, Lepage P, Gravel RA, Rosenblatt DS. The spectrum of mutations in the *MMAB* gene in patients with the *cblB* disorder of vitamin B12 metabolism. Gordon Research conference - Oxford, England, UK Poster. Sept. 2005.

Lerner-Ellis JP, Tirone JC, Pawelek PD, Dore C, Atkinson JL, Watkins D, Morel CF, Fujiwara TM, Moras E, Hosack AR, Dunbar GV, Antonicka H, Forgetta V, Dobson CM, Leclerc D, Gravel RA, Shoubridge EA, Coulton JW, Lepage P, Rommens JM, Morgan K, Rosenblatt DS. Identification of the gene responsible for methylmalonic aciduria and homocystinuria, *cblC* type. Gordon Research conference - Oxford, England, UK. Oral. Sept. 2005.

Lerner-Ellis JP, Dobson CM, Wai T, Watkins D, Tirone JC, Dore C, Lepage P, Gravel RA, Rosenblatt DS. Mutation and biochemical analysis of patients belonging to the cblB complementation class of vitamin B12-dependent methylmalonic aciduria. American Society of Human Genetics - Toronto Ontario, Can. Poster. *AJHG* Suppl. Nov; 75:336 (1821) 2004.

Lerner-Ellis JP, Dobson CM, Wai T, Watkins D, Tirone JC, Leclerc D, Dore C, Lepage P, Gravel RA, Rosenblatt DS. Mutations in the *MMAA* gene in patients with the *cblA*

disorder of Vitamin B_{12} metabolism. FASEB – 1-Carbon metabolism - Aspen, Colorado, USA. Poster. Aug. 2004.

Lerner-Ellis JP, Dobson CM, Wai T, Watkins D, Tirone JC, Dore C, Lepage P, Gravel RA, Rosenblatt DS. Mutation and biochemical analysis of patients belonging to the cblA complementation class of vitamin B12-dependent methylmalonic aciduria. American Society of Human Genetics - Hollywood, California, USA. Poster. *AJHG* Suppl. Nov; 73(5):459 (1696) 2003.

Lerner-Ellis JP, Dobson CM, Wai T, Watkins D, Tirone JC, Leclerc D, Dore C, Lepage P, Gravel RA, Rosenblatt DS. Mutation and biochemical analysis of patients belonging to the *cblA* complementation class of vitamin B_{12}-responsive methylmalonic aciduria.Gordon Research Conference (GRC) - Colby College, Main, USA. Poster. Sept. 2003.

APPENDIX B: Ethics Approval & Certificates

Centre universitaire de santé McGill
McGill University Health Centre

Bureau d'éthique de la recherche
Office of Research Ethics

September 18, 2006

Dr. D. Rosenblatt
Division of Medical Genetics
MGH
Room L3.319

REB NO. BMA 06-006

RE: Identification of the Gene Responsible for the cblF Form of Methymalonic Aciduria and Homocystinuria

Dear Dr. Rosenblatt:

The above-named study protocol received expedited review on September 13, 2006 and the study was found to be within ethical guidelines for conduct at the McGill University Health Centre, and will be entered into the minutes of the Research Ethics Board meeting of October 3, 2006. At the MUHC, sponsored research activities that require US federal assurance are conducted under Federal Wide Assurance (FWA) 00000840. Final approval of the protocol and English and French consent form dated September 11, 2006 was provided on September 13, 2006.

Below is a list of the Research Ethics Board members responsible for the review of the above named study. The names of the members are not disclosed for reasons of confidentiality. However, Dr. D. Rosenblatt, Principal Investigator and his research staff were not involved in the review process.

- 1 chair, MD
- 2 physicians
- 1 ethicist
- 1 legal member (non-affiliated)
- 1 nurse
- 2 patient community members (non-affiliated)
- 1 member of multidisciplinary committee (MDC)
- 1 pharmacist
- 1 research scientist

All research involving human subjects requires review at a recurring interval and the current study approval is in effect until September 12, 2007. An Application for Continuing Review must be submitted to the REB prior to the expiration of approval to comply with the regulation for continuing review of "at least once per year".

APPENDIX C: Permission to Reprint

JOHN WILEY & SONS CANADA, LTD.
PERMISSION REQUEST FORM

Complete this form as it pertains to your request and return it to the Permissions Department, John Wiley & Sons Canada, Ltd., 6045 Freemont Boulevard, Mississauga, ON, L5R 4J3; via facsimile to (416) 236-4446; via e-mail to mailto:sdam@wiley.com. Direct queries to (416) 236-4433 ext. 53033.

Requestor's Name: Jordan Lerner-Ellis

Date: Nov.16 2006

Address: 1650 Cedar ave MGH L3-319 Mtl. QC. H3G 1A4

Phone #: 514.934.1934 x42978 Fax #: 514.934.8536

Book Title and ISBN (including the ISBN will facilitate the permissions process):
Mutations in the MMAA gene in patients with the cblA disorder of vitamin B12 metabolism.
Hum Mutat. 2004 Dec;24(6):509-16. Erratum in: Hum Mutat. 2005 Mar;25(3):317.

Name(s) of Author(s): Lerner-Ellis JP, Dobson CM, Wai T, Watkins D, Tirone JC, Leclerc D, Dore C, Lepage P, Gravel RA, Rosenblatt DS.

Chapter Number/Page Number/Figure Numbers (for republication requests include photocopies of the original material): Entire paper

Number of copies to be made: 5 Number of Semesters:

Professor's Name: David S Rosenblatt

Course Name/Number:

Name of University/College/Organization: McGill University

Purpose of Reproduction: PhD Thesis

Title of Your Work, Publisher, Estimated First Print Run, Publication Date (if requesting rights to reproduce our material: The molecular characterization of inborn errors of vitamin B12 metabolism: cblA, cblB and cblC First Print ~Feb. 2007

Territory Requested (distribution: ex. World rights):

Electronic Requests Only:

Medium (ex. Intranet, Internet, CD-ROM):

Jordan Lerner-Ellis, Mr

From: Smith, Stephanie (ELS-OXF) [ST.Smith@elsevier.com] **Sent:** Fri 11/17/2006 6:25 AM
To: Jordan Lerner-Ellis, Mr
Cc:
Subject: RE: request
Attachments:

We hereby grant you permission to reproduce the material detailed below **in your thesis** at no charge subject to the following conditions:

1. If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies.

2. Suitable acknowledgment to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:

"Reprinted from Publication title, Vol number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier".

3. Reproduction of this material is confined to the purpose for which permission is hereby given.

4. This permission is granted for non-exclusive world **English** rights only. For other languages please reapply separately for each one required. Permission excludes use in an electronic form. Should you have a specific electronic project in mind please reapply for permission.

5. This includes permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission.

Yours sincerely

Steph Smith

Rights Assistant
Elsevier Ltd
The Boulevard
Langford Lane
Kidlington
Oxford OX5 1GB

From: Jordan Lerner-Ellis [mailto:jordan.lerner-ellis@mcgill.ca]
Sent: 16 November 2006 21:27
To: Rights and Permissions (ELS)
Cc: Dr. David S. Rosenblatt
Subject: request

Hi,
I would like to request permission to use the following two published articles in my PhD thesis.

1-

- **Lerner-Ellis JP, Gradinger AB, Watkins D, Tirone JC, Villeneuve A, Dobson CM, Montpetit A, Lepage P, Gravel RA, Rosenblatt DS.**

Mutation and biochemical analysis of patients belonging to the cblB complementation class of vitamin B12-dependent methylmalonic aciduria.
Mol Genet Metab. 2006 Mar;87(3):219-25. Epub 2006 Jan 10

2 -

- **Morel CF, Lerner-Ellis JP, Rosenblatt DS.**

Combined methylmalonic aciduria and homocystinuria (cblC): phenotype-genotype correlations and ethnic-specific observations.
Mol Genet Metab. 2006 Aug;88(4):315-21. Epub 2006 May 22.

Please let me know how to proceed.

Kind Regards,
Jordan

COPYRIGHT ASSIGNMENT nature publishing group npg

Manuscript #: NG - A17515 LERNER-ELLIS **Nature Genetics ("the Journal")**

Title of the contribution: Identification of the gene responsible for methylmalonic aciduria, cblC, type ("the Contribution")

Author(s): LERNER-ELLIS JP, TIRONE JC, PAWELEK PD, ("the Authors") DORE C, ATKINSON JL, WATKINS D, MOREL CF, FUJIWARA TM, MORAS E, HOSACK AR, DUNBAR GV, ANTONICKA H, FORGETTA V, DOBSON CM, LECLERC D, GRAVEL RA, SHOUCHOUG EA, COULTON JW, LEPAGE P, ROMMENS JM, MORGAN K, ROSENBLATT DS.

To: Nature America, Inc. trading as Nature Publishing Group ("NPG")

1. In consideration of NPG agreeing to publish the Contribution in the Journal the Author(s) hereby assign (where relevant by way of present assignment of future rights) to NPG all copyright and other intellectual property or related rights ("**Rights**") in the Contribution throughout the world for the full term of protection and all renewals, revivals and extensions.

2. Although ownership of all Rights in the Contribution is transferred to NPG, NPG hereby grants to the Author(s) a licence of the following non-exclusive, non-transferable rights, provided that (i) the rights must not be exercised before first publication of the Contribution in the Journal by the NPG; and (ii) when reproducing or referring to the Contribution or extracts from it the Author(s) must include an acknowledgement of NPG's ownership and first publication in the Journal [substantially in the following form: First published in [Journal name, issue number, volume number, year, DOI] © Nature Publishing Group, a division of Macmillan Publishers Limited]:

   a) They and any academic institution where they work at the time may reproduce the Contribution for the purpose of course teaching

   b) To reproduce, or to allow a third party publisher to reproduce the Contribution in whole or in part in any printed volume (book or thesis) authored by the Authors(s) subject to NPG's prior written approval to be granted at NPG's sole discretion; and

   c) To post a copy of the Contribution as accepted for publication after peer review (in Word or Text format) on the Author's own web site, or the Author's institutional repository, or the Author's funding body's archive, six months after publication of the printed or online edition of the Journal, provided that they also link to the Journal article on NPG's web site (eg through the DOI).

   d) To reuse figures or tables created by the Author(s) and contained in the Contribution in oral presentations and other works created by them.

3. The Author(s) warrant and represent that:

   a) The Author(s) are the sole authors of and sole owners of the Rights in the Contribution. If the Contribution includes materials of others, the Author(s) have either (i) obtained a written assignment from the owners of the copyright in all such materials to enable the Author(s) to grant the rights contained herein and attached copies of all such documents to this assignment: or (ii) identified such material on the list attached to this assignment with sufficient information to enable NPG to obtain such an assignment or such permissions necessary to exercise the rights granted herein;

   b) All of the facts contained in the Contribution are true and accurate;

   c) The Author who has signed this Agreement has full right, power and authority to enter into this Agreement on behalf of all of the Author(s);

   d) Nothing in the Contribution is obscene, defamatory, violates any right of privacy or publicity, infringes any intellectual property rights (including without limitation copyright, database rights or trade mark rights) or any other human, personal or other rights of any person or entity or is otherwise unlawful; and

   e) Nothing in the Contribution infringes any duty of confidentiality which any of the Author(s) may owe to anyone else or violates any contract, express or implied, of any of the Author(s), and all of the institutions in which work recorded in the Contribution was created have authorised publication of the Contribution.

4. The Author(s) hereby consent to the inclusion of electronic links from the Contribution to third-party material wherever it may be located.

This Agreement shall be governed by the laws of the State of New York without reference to its conflict of laws principles and by the Federal laws applicable therein.

Signed for and on behalf of the Authors [signature] **Date** SEPT 16 2005

Please print name DAVID ROSENBLATT

Address DEPARTMENT OF HUMAN GENETICS, MCGILL UNIVERSITY, MONTREAL, QUEBEC, CANADA H3G1A4

Nature America, Inc. (trading as Nature Publishing Group), principal place of business 345 Park Avenue South, New

APPENDIX D: Reprints of Articles

HUMAN MUTATION 24:509–516 (2004)

RESEARCH ARTICLE

Mutations in the *MMAA* Gene in Patients With the *cblA* Disorder of Vitamin B_{12} Metabolism

Jordan P. Lerner-Ellis,[1] C. Melissa Dobson,[6] Timothy Wai,[1] David Watkins,[1] Jamie C. Tirone,[1] Daniel Leclerc,[1] Carole Doré,[5] Pierre Lepage,[5] Roy A. Gravel,[6] and David S. Rosenblatt[1,2,3,4*]

[1]*Department of Human Genetics, McGill University, Montreal, Canada;* [2]*Department of Biology, McGill University, Montreal, Canada;* [3]*Department of Medicine, McGill University, Montreal, Canada;* [4]*McGill University Health Centre, Montreal, Canada;* [5]*McGill University and Genome Quebec Innovation Centre, Montreal, Canada;* [6]*Department of Biochemistry and Molecular Biology, University of Calgary, Alberta, Canada*

Communicated by Arnold Munnich

Mutations in the MMAA gene on human chromosome 4q31.21 result in vitamin B_{12}-responsive methylmalonic aciduria (*cblA* complementation group) due to deficiency in the synthesis of adenosylcobalamin. Genomic DNA from 37 *cblA* patients, diagnosed on the basis of cellular adenosylcobalamin synthesis, methylmalonyl–coenzyme A (CoA) mutase function, and complementation analysis, was analyzed for deleterious mutations in the MMAA gene by DNA sequencing of exons and flanking sequences. A total of 18 novel mutations were identified, bringing the total number of mutations identified in 37 *cblA* patients to 22. A total of 13 mutations result in premature stop codons; three are splice site defects; and six are missense mutations that occur at highly conserved residues. Eight of these mutations were common to two or more individuals. One mutation, c.433C>T (R145X), represents 43% of pathogenic alleles and a common haplotype was identified. Restriction endonuclease or heteroduplex diagnostic tests were designed to confirm mutations. None of the sequence changes identified in *cblA* patients were found in 100 alleles from unrelated control individuals. Hum Mutat 24:509–516, 2004. © 2004 Wiley-Liss, Inc.

KEY WORDS: vitamin B_{12}; cobalamin; methylmalonic aciduria; *cblA*; MMAA

DATABASES:
MMAA – OMIM: 607481, 251100 (*cblA*); GenBank: NM_172250.1, NT_016606.16

INTRODUCTION

Methylmalonic acidemia is a rare human disorder caused by a decreased activity of mitochondrial methylmalonyl–coenzyme A (CoA) mutase (MCM), one of two mammalian enzymes that utilize vitamin B_{12} (cobalamin, Cbl) derivatives. MCM catalyzes the adenosylcobalamin (AdoCbl)-dependent rearrangement of L-methylmalonyl-CoA to succinyl-CoA. This is an important intermediary step in the catabolism of branched chain amino acids and odd chain fatty acids via the tricarboxylic acid cycle. A defect in either MCM or the gene products involved in the conversion of exogenous Cbl to AdoCbl leads to the accumulation of methylmalonic acid in the blood and urine of affected individuals.

The *cblA* complementation class of inborn errors of cobalamin metabolism (MIM# 251100) is one of three known disorders that affect AdoCbl synthesis without also affecting the synthesis of methylcobalamin (MeCbl), a second Cbl derivative required for activity of the cytoplasmic enzyme methionine synthase [Rosenblatt and Fenton, 2001]. The gene responsible for *cblA* has been identified through the examination of prokaryotic gene arrangements and is called MMAA (MIM# 607481) [Dobson et al., 2002]. The identification of four mutations in five *cblA* patients confirmed the gene assignment. The precise role of the MMAA gene product is not known, but previous studies have suggested its involvement in either mitochondrial Cbl transport or reduction of cobalamin [Fenton and Rosenberg, 1978; Dobson et al., 2002]. More recently, a role for MMAA in the maintenance of MCM dimer stabilization and Cbl cofactor protection has been suggested [Korotkova and Lidstrom, 2004].

Individuals affected with *cblA* typically present with severe disease in infancy or early childhood and are prone to potentially life threatening acidotic crises [Rosenblatt and Fenton, 2001]. Cultured fibroblasts

Received 16 December 2003; accepted revised manuscript 27 May 2004.

*Correspondence to: David S. Rosenblatt, Department of Human Genetics, McGill University, Montreal, Quebec, Canada H3G1A4. E-mail: david.rosenblatt@mcgill.ca

Grant sponsors: Canadian Institutes for Health Research (CIHR); CIHR Group Grant in Medical Genetics, March of Dimes.

DOI 10.1002/humu.20104
Published online in Wiley InterScience (www.interscience.wiley.com).

from *cblA* patients have characteristic biochemical features, including: 1) reduced synthesis of AdoCbl from exogenous cyanocobalamin (CNCbl) with normal levels of methylcobalamin (MeCbl); 2) reduced ability to incorporate propionate into cellular macromolecules, an indirect measure of AdoCbl-dependent MCM activity; and 3) the ability to complement all other inborn errors of cobalamin metabolism in cell culture [Gravel et al., 1975; Fenton and Rosenberg, 1978; Willard et al., 1978; Watkins et al., 2000].

A total of 37 *cblA* patients were identified on the basis of AdoCbl synthesis, MCM function and complementation analysis. Our aim was to identify novel mutations in the MMAA gene in these patients by sequencing the exons and flanking regions.

MATERIALS AND METHODS

Patients

Sequencing of the MMAA gene (NM_172250.1) was carried out on a panel of 37 *cblA* patients and 50 anonymous controls. This includes five patients previously studied by Dobson et al. [2002]. Patient cell line numbers are listed in Table 1, along with the sex, race, and age of onset of these individuals, except for five patients for whom complete clinical information was not available. Consanguinity was reported in two individuals.

Fibroblast Studies

Diagnosis of *cblA* was based on assessment of cellular AdoCbl synthesis and MCM function, and was confirmed by complementation analysis [Watkins et al., 2000]. MCM function in intact fibroblasts was assessed by measuring incorporation of [^{14}C]propionate into acid-precipitable material. Cell lines were plated into 35-mm tissue culture dishes at a density of 4×10^5 cells per dish and incubated for 18 hr in Puck's F medium supplemented with 15% fetal bovine serum (FBS) and 100 μmol/l [^{14}C]propionate (New England Nuclear, www.perkinelmer.com), diluted with unlabeled propionate to give a final specific activity of 10 μCi/μmol. The assay was performed in the presence and absence of 3.75 μmol/l hydroxycobalamin (OHCbl). Macromolecules were precipitated with 5% trichloroacetic acid. The precipitated material was dissolved in 0.2 N sodium hydroxide and radioactivity was determined by liquid scintillation counting. All patient fibroblasts had decreased incorporation of label from [^{14}C]propionate into cellular macromolecules compared to control fibroblasts (Table 2).

TABLE 1. Summary of Mutations Found in *cblA* Patients

Cell line	Sex/race	Age of onset	Mutation 1	Predicted change	Mutation 2	Predicted change
WG1449	F/W	Neonatal	c.64C>T	R22X	c.266T>C	L89P
WG1588	F/B	6 weeks	c.64C>T	R22X	c.653G>A	G218E
WG2578	F/W	1 week	c.161G>A	W54X	c.266T>C	L89P
WG3080	F/W	1 week	c.260_267dup	Frameshift	c.592_595del	Frameshift
WG1776	F/W	7 months	c.283C>T	Q95X	c.439+1_4del	Splice site
WG1798	M/W	7 months	c.283C>T	Q95X	c.970-2 A>T	Splice site
WG3009	F/W	3 months	c.387C>A	Y129X	c.387C>A	Y129X
WG2019	M/unknown	Neonatal	c.433C>T	R145X	c.433C>T	R145X
WG1796	F/W	2 weeks	c.433C>T	R145X	c.433C>T	R145X
WG2653	F/W	2 weeks	c.433C>T	R145X	c.433C>T	R145X
WG1660	M/W	6 months	c.433C>T	R145X	c.433C>T	R145X
WG3038	M/W	7 months	c.433C>T	R145X	c.433C>T	R145X
WG1802	M/W	12 months	c.433C>T	R145X	c.433C>T	R145X
WG1761	F/W	14 months	c.433C>T	R145X	c.433C>T	R145X
WG2529	M/W	18 months	c.433C>T	R145X	c.433C>T	R145X
WG2037	M/W	4.5 years	c.433C>T	R145X	c.433C>T	R145X
WG3039	F/W	10 years	c.433C>T	R145X	c.433C>T	R145X
WG2893	Unknown	4 weeks	c.433C>T	R145X	c.450_451ins	Frameshift
WG2185	F/W	11 months	c.433C>T	R145X	c.450_451ins	Frameshift
WG3003	F/W/A	9 months	c.433C>T	R145X	c.503del	Frameshift
WG2664	M/W	4 days	c.433C>T	R145X	c.592_595del	Frameshift
WG2014	M/W	3 months	c.433C>T	R145X	c.592_595del	Frameshift
WG2922	M/W	5 months	c.433C>T	R145X	c.592_595del	Frameshift
WG1516	M/W	5 months	c.433C>T	R145X	c.592_595del	Frameshift
WG2230	F/B	12 months	c.433C>T	R145X	c.742C>T	Q248X
WG3084	M/W	9 months	c.433C>T	R145X	c.1076G>A	R359Q
WG1191	Unknown	Unknown	c.434G>A	R145Q	c.434G>A	R145Q
WG1518	M/W	6 months	c.440G>A	E147G	c.450_451ins	Frameshift
WG1192	Unknown	Unknown	c.592_595del	Frameshift	c.592_595del	Frameshift
WG1943	M/W	2 months	c.620A>G	Y207C	c.620A>G	Y207C
WG2188	F/Unknown	12 months	c.653G>A	G218E	c.1089_1090del	Frameshift
WG2623	M/W	4 days	c.733+1G>A	Splice site	c.733+1 G>A	Splice site
WG2627	M/W	3.75 years	c.959G>A	W320X	c.959G>A	W320X
WG2063	F/W	5 days	c.988C>T	R330X	c.988C>T	R330X
WG2704	M/W	6.5 years	c.1076G>A	R359Q	c.1076G>A	R359Q
WG2882	F/W	6 months	c.433C>T	R145X	ND	
WG1411	M/W	26 months	c.433C>T	R145X	ND	

Mutations in the *MMAA* gene (NT_016606.16) in patients with *cblA* are summarized. DNA mutation numbering is based on cDNA sequence: +1 corresponds to the A of the ATG translation initiation codon. In 35 patients, two mutations were identified, and in two patients, one mutation was identified. Consanguinity was reported in cell lines WG2627 & WG2037. Cell lines are arranged sequentially by the position of the first mutation from 5′ to 3′, and then by the age of onset, in patients where two mutations were identified. See text for mutation details.
F, female; M, male; W, white; B, black; A, Asian; ND, no mutation detected.

TABLE 2. *cblA* Patient Fibroblast Cobalamin Distributions and Propionate Incorporation Profiles

Cell line	AdoCbl (% of total intracellular cobalamin)	Prop. Inc. w/o OHCbl[a]	Prop. Inc. w/OHCbl[b]
Control[c]	15.3 ±4.2 (n = 3)	11 ±4	11 ±4
WG1449	3.4	2.3	13.0
WG1588	3.9	1.9	7.2
WG2578	6.1	0.7	3.3
WG3080	3.5	0.6	2.4
WG1776	6.6	2.3	4.6
WG1798	5.2	2.9	2.1
WG3009	1.5	1.3	2.2
WG2019	3.7	1.2	5.0
WG1796	3.7	1.5	4.4
WG2653	4.3	0.5	2.4
WG1660	2.8	2.5	6.6
WG3038	1.8	1.0	4.0
WG1802	3.5	0.4	3.3
WG1761	4.4	1.9	6.8
WG2529	1.5	0.5	3.0
WG2037	2.2	2.6	5.4
WG3039	2.8	3.0	5.9
WG2893	1.5	1.2	4.7
WG2185	5.0	1.0	2.0
WG3003	3.5	1.6	3.4
WG2664	3.1	1.2	6.7
WG2014	7.5	1.2	7.1
WG2922	1.4	0.9	2.3
WG1516	4.6	0.7	2.9
WG2230	4.0	1.9	4.7
WG3084	3.1	0.7	3.7
WG1191	5.0	0.7	N/D
WG1518	7.0	4.5	11.2
WG1192	5.0	1.3	N/D
WG1943	6.8	3.4	9.0
WG2188	3.4	0.9	2.6
WG2623	1.7	0.9	3.2
WG2627	1.9	0.5	3.5
WG2063	2.8	3.3	7.0
WG2704	6.1	0.9	3.0
WG2882	3.2	1.1	2.6
WG1411	2.3	1.6	6.1

[a]Prop. Inc. w/o OHCbl: Incorporation of [^{14}C]propionate (nmoles/mg protein/18 hrs) without addition of OHCbl.
[b]Prop. Inc. w/OHCbl: Incorporation of [^{14}C]propionate (nmoles/mg protein/18 hrs) with the addition of 3.75μM OHCbl.
[c]Control values (mean ±SD) are based on 12 determinations in 3 different controls.
N/D, not done.

Cobalamin distributions were determined by growing cells in 25 pg/ml [^{57}Co]CNCbl (Amersham, www5.amershambiosciences.com or MP Biomedicals, www.mpbio.com) for 4 days, followed by extraction of labeled cobalamins in darkness in absolute ethanol at 85°C. Intracellular cobalamins were separated by high performance liquid chromatography (HPLC) as previously described [Rosenblatt et al., 1984]. All patient fibroblast lines had decreased synthesis of AdoCbl from [^{57}Co]CNCbl compared to controls (Table 2).

DNA Sequencing

DNA was extracted from patient and control fibroblasts using the Qiagen genomic DNA extraction kit for cultured cells. MMAA exons 1–3 and flanking sequences were amplified by PCR using primers listed in Table 3 (primers were designed using Primer 3.0 software available online at www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and exons 4–7 were amplified using primers described by Dobson et al. [2002]. PCR products were purified with Montage PCR$_{96}$ filter plates (Millipore, www.millipore.com). Purified PCR products were used for 10 μl sequencing reactions made up of 2 μl PCR product, 1 μl of BigDye Terminator Cycle Sequencing Version 2.0 or Version 3.0 (Applied Biosystems, www.appliedbiosystems.com), 1.5 μl of 5 × buffer, 5 μl H_2O, and 0.5 μl (1 μM final) of sense or anti-sense primer. Amplicons were sequenced in both forward and reverse directions. Products were analyzed on an ABI3700 automated DNA sequencer (Applied Biosystems). Gel files were processed using Sequence Analysis software (PE Applied Biosystems) and were assembled and analyzed using the Phred/Phrap/Consed System [Ewing et al., 1998; Gordon et al., 1998]. DNA mutation numbering is based on cDNA sequence with +1 corresponding to the A of the ATG translation initiation codon.

Restriction Endonuclease and Heteroduplex Analysis

MMAA sequence changes identified by sequence analysis were confirmed by restriction endonuclease or heteroduplex analysis. Restriction endonucleases were purchased from New England Biolabs (www.neb.com). A total of 10 sequence changes resulted in either the creation or loss of a naturally occurring restriction site. Five sequence changes were confirmed by analysis using artificially created restriction sites. Digestions were performed under conditions recommended by the enzyme manufacturer. Five sequence changes (one duplication and four deletions) detected by sequencing were confirmed by heteroduplex analysis of PCR products. PCR products were denatured for 4 minutes at 94°C prior to electrophoresis on 8% polyacrylamide gels (29:1 acrylamide:bisacrylamide). Table 3 describes the primers and restriction enzymes used to detect sequence changes.

RESULTS

Two mutations in the MMAA gene coding sequence and/or flanking region were identified in 35 *cblA* patients and a single mutation was identified in two patients. None of these mutations was detected in 100 control alleles. Mutations were identified in exons 2–7. No mutations were detected in exon 1, which is likely untranslated due to the presence of an in-frame stop codon that precedes the first in-frame ATG in exon 2.

Novel MMAA Mutations

Mutations are listed in Table 1 and Figure 1. Details of mutation confirmation analysis are described in Table 3.

c.64C>T (R22X). This C>T sequence change at position c.64 results in a nonsense mutation by changing an arginine codon at position 22 to a stop codon in exon 2 and is common to both WG1449 and WG1588 cell lines in heteroallelic form.

c.161G>A (W54X). Cell line WG2578 is heteroallelic for a G>A sequence change at c.161, which results in the substitution of a stop signal for tryptophan at codon 54 in exon 2.

c.266T>C (L89P). A T>C nucleotide change at position c.266 in exon 2 results in the substitution of proline for leucine at codon 89. The leucine residue is conserved between mouse and human and occurs within the mitochondrial leader sequence, which is not present in orthologous polypeptides of *Esherichia coli*, *Mycobacterium tuberculosis*, and *Archeoglobus fulgidus*. This change was detected in two cell lines, WG1449 and WG2578, in heteroallelic form.

c.387C>A (Y129X). Cell line WG3009 is homoallelic for a nonsense mutation in exon 2 at position c.387

TABLE 3. *MMAA* Mutation Confirmation

Mutation or exon	Heteroduplex or enzyme	Primers		PCR product size (bp)	Fragment sizes[a]	
		5′-3′ Sense	5′-3′ Antisense		Wild-type	Mutant
c.64C>T[f]	*NlaIV*	CCTAAAAGGCCTTTTAAGAGCACGGTTC[b]	2R[c]	412	412	386/26
c.161G>A[g]	*FokI*	2F[c]	2R[c]	515	298/<15	515
c.266T>C[f]	*BslI*	2F[c]	2R[c]	515	515	339/176
c.260_267dup	Heteroduplex	2F[c]	2R[c]	515	N/A	N/A
c.282C>T[g]	*StyI*	2F[c]	2R[c]	515	350/165	515
c.387C>A[f]	*MseI*	2F[c]	2R[c]	515	170/117/82/66/50/30	117/107/82/66/63/50/30
c.433C>T[g]	*TaqI*	GCCATGGAGGGAGTCTTCTC	TGGGATCCAGAGCAAGATTC	943	529/169/149/96	625/169/149
c.434C>T[g]	*TaqI*	GCCATGGAGGGAGTCTTCTC	TGGGATCCAGAGCAAGATTC	943	529/169/149/96	625/169/149
c.439+1_4del	Heteroduplex	GCCATGGAGGGAGTCTTCTC	TGGGATCCAGAGCAAGATTC	943	N/A	N/A
c.440G>A[f]	*Tsp509I*	GAACCCAGGGTGTTTCCTTC	CCTTCCTTTAGCGAGACCAA	457	194/104/93/68/52/45/30/11	194/93/78/68/52/45/30/26/11
c.450_451ins	N/A[d]	3F[c]	3R[c]	586	N/A	N/A
c.503del	Heteroduplex	3F[c]	3R[c]	586	N/A	N/A
c.592-595del	Heteroduplex	4F[c]	4R[c]	507	N/A	N/A
c.620A>G[f]	*SphI*	4F[c]	4R[c]	507	507	384/123
c.653G>A[g]	*MnlI*	4F[c]	4R[c]	507	266/137/64/28/12	278/137/64/28/24
c.733+1G>A[g]	*HphI*	4F[c]	4R[c]	507	259/159/89	418/89
c.742C>T[g]	*PleI*	GTGATTTACAATTTCAGGTGTGAGT[b]	CCTTTGATTCAGGTATTTTAGCC	250	220/30	250
c.959G>A	N/A[d]	CGCAAACGTTCACAAGACTG[e]	ATGACTACACACTCCGTCTTTGTC[e]	185	N/A	N/A
c.970-2A>T[g]	*Eco0109I*	7F[c]	TCTCCACTTCGGGCAGAAATACGAAGGACC[b]	117	88/29	117
c.988C>T[g]	*ApaI*	TCGATAGGTAATTCGTATTTGGGCC[b]	GAATCTGTTCCCGGACTGTG	206	181/25	206
c.1076G>A[g]	*BslEI*	7F[c]	CAAACTTTCTGTTGCTTCGGT[b]	210	186/24	210
c.1089_1090del	Heteroduplex	7F[c]	7R[c]	352	N/A	N/A
Exon 1	N/A	TCACCGGTCTGTCAAACGTA	TCCTGCCTGTCCACACATATC	707	N/A	N/A
Exon 2	N/A	GCCATGGAGGGAGTCTTCTC	TGGGATCCAGAGCAAGATTC	943	N/A	N/A
Exon 3	N/A	GAACCCAGGGTGTTTCCTTC	CCTTCCTTTAGCGAGACCAA	457	N/A	N/A

[a]Heteroallelic restriction cuts would have both fragment sizes observed.
[b]The underlined nucleotides denote changes made in the PCR primer to incorporate an artificial restriction site.
[c]Primer sequence is from Dobson et al. (2002).
[d]This sequence change was confirmed by sequence analysis only.
[e]Second primer used to confirm mutation by sequence analysis.
[f]The mutation results in the creation of a restriction enzyme site.
[g]The mutation results in the destruction of a restriction enzyme site.
N/A, not applicable.

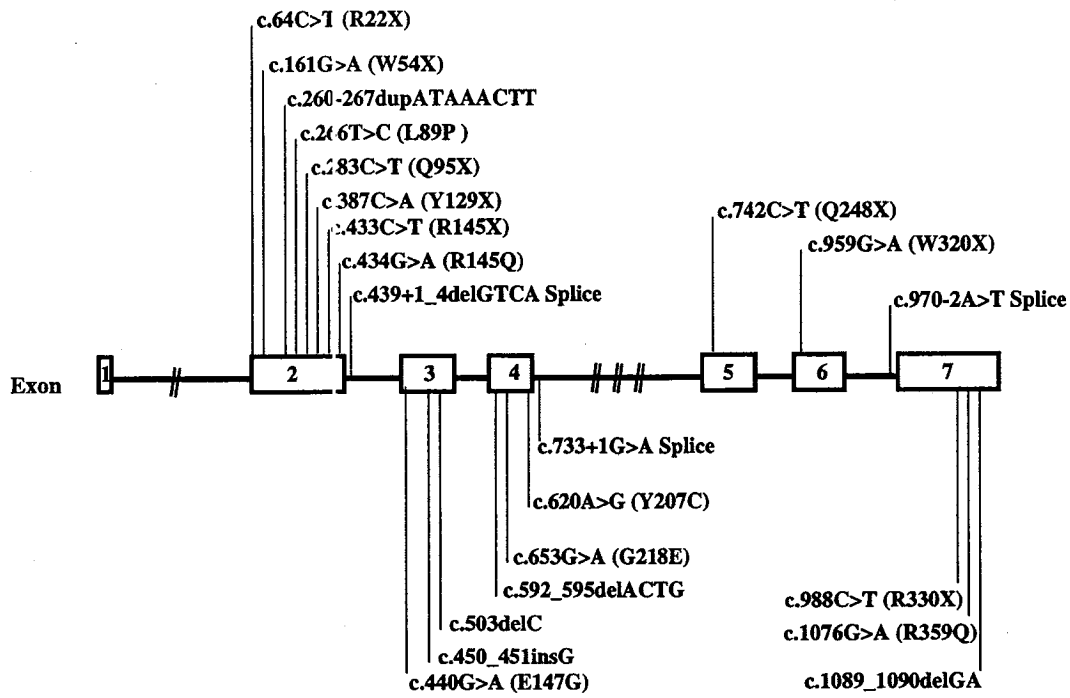


FIGURE 1. **The exon structure of the *MMAA* gene (NT_016606.16) and the position of mutations identified with the corresponding amino acid change. The introns are drawn to scale except where "//" denotes a region longer than indicated. See Dobson et al. [2002] for structural organization of the *MMAA* gene.**

due to a C>A nucleotide change. This results in the replacement of tyrosine by a stop codon at amino acid 129.

c.433C>T (R145X). This C>A sequence change occurs in exon 2 at position c.433 and it accounts for 31 of 72 (~43%) pathogenic alleles in this panel of *cblA* patients. Arginine is converted to a stop signal at codon 145. A total of 10 patients were homoallelic and 11 patients were heteroallelic for this mutation (Table 1). This mutation was not detected in 132 control alleles examined.

c.434G>A (R145Q). Cell line WG1191 is homoallelic for a G>A nucleotide change at position c.434. Arginine is converted to a glutamine at codon 145. This residue is conserved between human, mouse, *E. coli* and *M. tuberculosis*, but not with *A. fulgidus*, which has valine at this position, five amino acids upstream of the Walker A motif.

c.439+1_4delGTCA. This deletion results in a splice site defect. Four bases, GTCA, are deleted at the exon 2 donor splice site. Cell line WG1776 is heteroallelic for this sequence change.

c.440G>A (E147G). The first nucleotide of exon 3, c.440, is converted from G>A, resulting in the substitution of glycine for glutamic acid at amino acid position 147. This amino acid is conserved between human, mouse, *E. coli*, *M. tuberculosis*, and *A. fulgidus*, and it is three amino acids upstream of the Walker A motif. Cell line WG1518 is heteroallelic for this sequence change.

c.450_451insG. An insertion of a guanine nucleotide at cDNA position 450–451 of exon 3 results in a frame shift mutation and creation of a stop signal downstream at amino acid position 169. Cell lines WG1518, WG2185, and WG2893 are heteroallelic for this insertion.

c.503delC. A cytosine deletion was detected at position c.503 in exon 3. A truncated protein product is predicted from this mutation. Cell line WG3003 is heteroallelic for this mutation.

c.653G>A (G218E). Cell lines WG1588 and WG2188 are both heteroallelic for a point mutation at position c.653 in exon 4, a G>A change that results in a glycine to a glutamic acid substitution at codon 218. The glycine residue is highly conserved between human, mouse, *E. coli*, *M. tuberculosis*, and *A. fulgidus*. This residue is located between the predicted Mg^{2+} binding site and Walker B motif of the polypeptide chain.

c.733+1G>A. Cell line WG2623 is homoallelic for a G>A nucleotide change at splice site position c.733+1 in intron 4. Disruption of MMAA transcript processing is predicted.

c.742C>T (Q248X). Sequence analysis of DNA from cell line WG2230 revealed a heteroallelic point mutation at position c.742 in exon 5. A C>T nucleotide change results in the substitution of a stop signal for a glutamine residue at codon 248.

c.959G>A (W320X). A homoallelic nonsense mutation was found in cell line WG2627 at codon 320 of exon 6 as the result of a G>A sequence change at position c.959.

c.970–2A>T. Cell line WG1798 is heteroallelic for a splice site mutation at position −2 of the intron 6–exon 7 boundary.

c.988C>T (R330X). The WG2063 cell line is homoallelic for a C>T nucleotide change at position c.988 in exon 7, resulting in the creation of a nonsense mutation at codon 330. The mutation is 37 amino acids downstream of a predicted GTP binding site.

c.1076G>A (R359Q). Sequence analysis of DNA from the WG3084 cell line revealed a heteroallelic G>A change at nucleotide position c.1076 in exon 7. Cell line WG2704 is homoallelic for this sequence change. This alteration results in the conversion of the normal arginine codon to a glutamine codon at position 359, resulting in a charge change at this site. The c.1076 guanine nucleotide is highly conserved between human, mouse, *E. coli*, *M. tuberculosis*, and *A. fulgidus*.

c.1089_1090delGA. This mutation occurs in exon 7. Guanine and adenine are deleted at positions c.1089_1090, resulting in a frameshift. Cell line WG2188 is heteroallelic for this mutation.

MMAA mutations (c.260_267dupATAAACTT, c.283C>T (Q95X), c.592_595delACTG and c.620A>G (Y207C)) previously identified by Dobson et al. [2002] were confirmed in the originally described patients and tested for in the remainder of the 32 patient cell lines. The nomenclature of mutations, c.260_267dupATAAACTT and c.592_595delACTG, have been changed from their original designation [Dobson et al., 2002] to adhere to current guidelines [den Dunnen and Antonarakis, 2000]. The c.283C>T (Q95X) mutation, initially reported in cell line WG1776, was also observed in one additional cell line: WG1798. The c.592_595delACTG frameshift mutation was originally detected in cell lines WG1192, WG2014, and WG3080. This sequence change was also observed in three additional cell lines: WG2664, WG1516, and WG2922. Cell line WG1192 is homoallelic and the five other cell lines are heteroallelic.

SNPs

Five single nucleotide polymorphisms (SNPs) were observed in intronic regions: c.1–56A>G (rs4835011), c.439+108A>G (rs4835012), c.734–74G>A (rs11721510), c.820–169T>C (rs2279717), and c.820–110A>G. SNPs c.734–74G>A and c.820–110A>G were not previously documented. Table 4 lists the allelic frequencies of SNPs in patients and controls. Interestingly, c.820–110A>G was not found in any of the controls from our panel, but is present in 31 out of 74 patient alleles, all in patients with the c.433C>T mutation. A sequence change at position c.1089G>C (rs2270655), which results in a Q363H amino acid change, was identified in 4 out of 100 control alleles in heteroallelic form. A silent sequence change, c.747G>A (S249S), was detected in homoallelic form in cell line WG2627, and in 6 out of 100 control alleles in heteroallelic form.

TABLE 4. SNP Allelic Frequency*

SNPs	Frequency in patient alleles	Frequency in normal alleles
c.1-56A>G (rs4835011)	5/56 (8.9%)	13/182 (7.1%)
c.439+108A>G (rs4835012)	3/56 (5.4%)	30/182 (16.5%)
c.734-74G>A (rs11721510)	11/74 (14.9%)	14/100 (14%)
c.747G>A (S249S)	2/74 (2.7%)	6/100 (6%)
c.820-169T>C (rs2279717)	12/74 (16.2%)	55/100 (55%)
c.820-110A>G	31/74 (41.9%)	0/100 (0%)
c.1089G>C (Q363H) (rs2270655)	0/74 (0%)	4/100 (4%)

*Allelic frequency of SNPs within the *MMAA* gene in patients and controls as observed by sequence analysis in this study.

Sequence Change	c.433C>T		c.592_595del		c.433C>T/ c.592_595del	
c.1-56A>G	A	A	A	A	A	A
c.439+108A>G	A	A	A	A	A	A
c.734-74G>A	G	G	G	G	G	G
c.820-169T>C	T	T	T	T	T	T
c.820-110A>G	G	G	A	A	G	A

FIGURE 2. **Haplotypes of patients with the c.433C>T (R145X) and c.592_595delACTG mutations. Haplotypes were constructed using the genotypes of patients for the polymorphisms: c.1–56A>G, c.439+108A>G, c.734–74G>A, c.820–169T>C, and c.820–110A>G within the *MMAA* gene. Results are shown for patients that were homozygous for either c.433C>T (n=10) or c.592_595del (n=1), or heterozygous for both (n=4). Patients carrying these mutations are listed in Table 1.**

Haplotype Analysis

Haplotypes were constructed from genotype data for patients homoallelic for the c.433C>T and c.592_595delACTG mutations using five SNP markers detected by sequencing analysis (c.1–56A>G (rs4835011), c.439+108A>G (rs4835012), c.734–74G>A (rs11721510), c.820–169T>C (rs2279717), and c.820–110A>G). These markers span 14,801 bp from intron 1 to intron 5. A common haplotype was observed in patients with each of these mutations (Fig. 2).

DISCUSSION

In this study, we report the identification of 18 novel mutations in the MMAA gene of *cblA* patients. Four previously identified disease-causing mutations in MMAA reported in five *cblA* patients [Dobson et al., 2002] were confirmed, and second mutations were identified in two of these patients (Cell lines WG1776 and WG2014). In total, 22 mutations have been identified: 13 result in premature stop codons, including three deletions, one insertion, and one duplication; three are splice site defects; and six are missense mutations that occur at highly conserved residues. Two mutations

were identified in 35 patients, consistent with autosomal recessive inheritance of the *cblA* disorder, and one mutation was identified in two patients. None of the sequence changes identified in *cblA* patients was found in a minimum of 100 alleles from unrelated control individuals.

Common Mutations

Several mutations were identified in more than one individual. The c.433C>T (R145X) mutation was identified in 21 patients; this sequence change accounts for ~43% of pathogenic alleles in this panel of *cblA* patients. All patients homozygous for the c.433C>T mutation were also homozygous at five SNPs within the MMAA gene (Fig. 2), allowing definition of a common haplotype associated with this mutation. The c.820–110A>G SNP was seen only in patients carrying the c.433C>T mutation and not detected in any controls from our sample. Genotypes of patients heterozygous for the c.433C>T mutation were also consistent with the occurrence of c.433C>T on the same haplotype. This mutation was observed primarily in patients of European ancestry, but was also detected in heterozygous form in a Black individual and one of mixed Asian and European origin. These data suggest a common ancestor for the c.433C>T mutation.

The c.592_595delACTG mutation was identified in six individuals, five of whom were of European origin; information on the ethnic background of the sixth patient was unavailable. Cell line WG1192 is homozygous for this deletion and was also homozygous across five intronic SNPs (Fig. 2), allowing inference of a haplotype associated with this mutation. The genotypes of the remaining five patients who carry this deletion in heterozygote form are consistent with the mutation being on the same haplotype as in cell line WG1192. Four patients were compound heterozygote for c.592_595delACTG and c.433C>T mutations. Genotypes of these individuals were consistent with the haplotypes defined in patients homozygous for these mutations.

Other common mutations identified in more than one individual include c.64C>T (R22X), c.266T>C (L89P), c.653G>A (G218E), c.283C>T (Q95X), c.450_451insG, and c.1076G>A (R359Q). Further study will be necessary to determine if common mutations are being transmitted on the same genetic background.

Genotype–Phenotype Correlation

No direct correlations could be made between the mutations identified in the MMAA gene and clinical severity or the degree of impairment of cobalamin metabolism observed in tissue culture studies. Clinically, there was not much variation in presentation among the *cblA* patients in the present study. All patients presented during infancy or childhood with signs of metabolic acidosis. Variation in age of onset may be at least partly explained by environment, since in several cases, patients with childhood onset were reported to be self-limiting their protein intake prior to diagnosis. The age of onset of patients homozygous for the c.433C>T mutation varied from the neonatal period to 10 years of age. This suggests that environmental factors, genetic modifiers, or both, may affect the clinical presentation of the *cblA* disorder. Interestingly, of all the vitamin B_{12} inborn errors of metabolism, the *cblA* patients are the most responsive clinically to vitamin B_{12} supplementation.

The MMAA gene has four sequence motifs that define the ArgK subfamily of G3E GTPases, including the Walker A motif, a Mg^{2+}-binding aspartate residue, the Walker B motif, and a GTP-binding motif, as described by Leipe et al. [2002]. The function of the MMAA gene product is not known and it is currently not possible to predict functional effects of missense mutations. A role for the MMAA gene product has recently been suggested. A MMAA homologue, meaB from *Methylobacterium extorquens*, was hypothesized to protect MCM from irreversible inactivation. MeaB was shown to bind MCM and suggested to stabilize the dimer form of the enzyme and/or to protect bound cofactor from attack by oxygen, water, and highly reactive radical intermediates [Korotkova and Lidstrom, 2004]. Further investigation of defects in MMAA will be critical to our understanding of the human MMAA gene product and to its role in patients with the *cblA* disorder.

ACKNOWLEDGMENTS

We thank the clinicians who provided patient samples and clinical information, Nora Matiaszuk for complementation analysis, Gail Dunbar for growth of patient fibroblasts, Brian Gilfix, Maria Galvez, Antonia Klitorinos, Angela Hosack, Lisa Worgan, Kirsten Niles, and Emily Moras for assistance, and Vince Forgetta for quality control of sequence data. This is a publication of the Hess B. and Diane Finestone Laboratory in memory of Jacob and Jenny Finestone.

REFERENCES

den Dunnen JT, Antonarakis SE. 2000. Mutation nomenclature extensions and suggestions to describe complex mutations: a discussion. Hum Mutat 15:7–12.

Dobson CM, Wai T, Leclerc D, Wilson A, Wu X, Doré C, Hudson T, Rosenblatt DS, Gravel RA. 2002. Identification of the gene responsible for the *cblA* complementation group of vitamin B_{12}-responsive methylmalonic acidemia based on analysis of prokaryotic gene arrangements. Proc Natl Acad Sci USA 99:15554–15559.

Ewing B, Hillier L, Wendl M, Green P. 1998. Basecalling of automated sequencer traces using phred I. Accuracy assessment. Genome Res 8:175–185.

Fenton WA, Rosenberg LE. 1978. Genetic and biochemical analysis of human cobalamin mutants in cell culture. Annu Rev Genet 12:223–248.

Gordon D, Abajian C, Green P. 1998. Consed: a graphical tool for sequence finishing. Genome Res 8:175–185.

Gravel RA, Mahoney MJ, Ruddle FH, Rosenberg LE. 1975. Genetic complementation in heterokaryons of human fibroblasts

defective in cobalamin metabolism. Proc Natl Acad Sci USA 72:3181–3185.

Korotkova N, Lidstrom ME. 2004. MeaB is a component of the methylmalonyl-CoA mutase complex required for protection of the enzyme from inactivation. J Biol Chem 279:13652–13658.

Leipe DD, Wolf YI, Doonin EV, Aravind L. 2002. Classification and evolution of P-loop GTPases and related ATPases. J Mol Biol 317:41–72.

Rosenblatt DS, Cooper BA, Pottier A, Lue-Shing H, Matiaszuk N, Grauer K. 1984. Altered vitamin B_{12} 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.

Rosenblatt D, Fenton WA. 2001. Inherited disorders of folate and cobalamin transport and metabolism. In: Scriver CR, Beaudet AL, Sly WS, Valle D, Childs B, Kinzler KW, Vogelstein B, editors. The metabolic and molecular bases of inherited disease, 8th edition. New York: McGraw-Hill. p 3897–3933.

Watkins D, Matiaszuk N, Rosenblatt DS. 2000. Complementation studies in the *cblA* class of inborn error of cobalamin metabolism: evidence for interallelic complementation and for a new complementation class (*cblH*). J Med Genet 37:510–513.

Willard HF, Mellman IS, Rosenberg LE. 1978. Genetic complementation among inherited deficiencies of methylmalonyl-CoA mutase activity: evidence for a new class of human cobalamin mutant. Am J Hum Genet 30:1–13.

Available online at www.sciencedirect.com
SCIENCE DIRECT
Molecular Genetics and Metabolism 87 (2006) 219–225
Molecular Genetics and Metabolism
www.elsevier.com/locate/ymgme

Mutation and biochemical analysis of patients belonging to the *cblB* complementation class of vitamin B_{12}-dependent methylmalonic aciduria

Jordan P. Lerner-Ellis [a,f], Abigail B. Gradinger [a,f], David Watkins [a,f], Jamie C. Tirone [a,f,†], Amélie Villeneuve [b], C. Melissa Dobson [c], Alexandre Montpetit [b], Pierre Lepage [b], Roy A. Gravel [c], David S. Rosenblatt [a,d,e,f,*]

[a] *Department of Human Genetics, McGill University, Montreal, Que., Canada*
[b] *McGill University, Genome Quebec Innovation Centre, Montreal, Que., Canada*
[c] *Department of Biochemistry and Molecular Biology, University of Calgary, Calgary, Alta., Canada*
[d] *Department of Biology, McGill University, Montreal, Que., Canada*
[e] *Department of Medicine, McGill University, Montreal, Que., Canada*
[f] *McGill University Health Centre, Montreal, Que., Canada*

Received 1 October 2005; received in revised form 16 November 2005; accepted 18 November 2005
Available online 10 January 2006

Abstract

Methylmalonic aciduria, *cblB* type (OMIM 251110) is an inborn error of vitamin B_{12} metabolism that occurs due to mutations in the *MMAB* gene. *MMAB* encodes the enzyme ATP:cobalamin adenosyltransferase, which catalyzes the synthesis of the coenzyme adenosylcobalamin required for the activity of the mitochondrial enzyme methylmalonyl CoA mutase (MCM). MCM catalyzes the isomerization of methylmalonyl CoA to succinyl CoA. Deficient MCM activity results in methylmalonic aciduria and a susceptibility to life-threatening acidotic crises. The *MMAB* gene was sequenced from genomic DNA from a panel of 35 *cblB* patients, including five patients previously investigated. Nineteen *MMAB* mutations were identified, including 13 previously unknown mutations. These included 11 missense mutations, two duplications, one deletion, four splice-site mutations, and one nonsense mutation. None of these mutations was identified in 100 control alleles. Most of the missense mutations (9/11) were clustered in exon 7; many of these affected amino acid residues that are part of the probable active site of the enzyme. One previously described mutation, c.556C > T (p.R186W), was particularly common, accounting for 33% of pathogenic alleles. It was seen almost exclusively in patients of European background and was typically associated with presentation in the first year of life.

Keywords: Cobalamin; Vitamin B_{12}; Cobalamin adenosyltransferase; *cblB*; *MMAB*; Methylmalonic aciduria; Inborn error of metabolism

Introduction

Methylmalonic aciduria, *cblB* type (OMIM 251110) is an inborn error of cobalamin (Cbl, vitamin B_{12}) metabolism characterized by accumulation of methylmalonic acid in blood and urine [1]. Patients typically present within the first year of life with lethargy, failure to thrive, recurrent vomiting, dehydration, respiratory distress, and hypotonia, and are prone to life-threatening acidotic crises; however, several patients have presented in childhood or adolescence [2]. Accumulation of methylmalonic acid results from decreased activity of the mitochondrial enzyme methylmalonyl CoA mutase (MCM). This occurs either as the result of mutations affecting the *MUT* gene (*mut* complementation class), which encodes MCM, or mutations affecting genes encoding enzymes involved in the synthesis of adenosylcobalamin

* Corresponding author. Fax: +1 514 934 8536.
E-mail address: david.rosenblatt@mcgill.ca (D.S. Rosenblatt).
† Deceased.

1096-7192/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.ymgme.2005.11.011

(AdoCbl), which is required for the activity of MCM (the *cblA* and *cblB* complementation classes). MCM catalyzes the isomerization of methylmalonyl CoA to succinyl CoA. This intermediary step is critical for normal catabolism of isoleucine, methionine, threonine, valine, odd chain fatty acids, and cholesterol via the tricarboxylic acid cycle, a process that occurs in the mitochondria.

The genes underlying the *cblA* and *cblB* disorders have been identified by the analysis of prokaryotic gene arrangement. The function of the product of the *MMAA* gene, which is mutated in patients with the *cblA* disorder [3], remains unknown; roles in reduction of cob(II)alamin [4], as an accessory in mitochondrial membrane cobalamin transport [2], or in maintenance of MCM dimer stabilization [5] have been suggested. The *MMAB* gene, on chromosome 12q24.1, codes for the mitochondrial enzyme ATP:cobalamin adenosyltransferase (ATR), which catalyzes transfer of an adenosyl group from ATP to cob(I)alamin to form AdoCbl [6]. Deleterious mutations in *MMAB* were identified in six *cblB* patients. The gene was identified independently by Leal et al. [7], who identified and isolated clones from a bovine liver cDNA expression library that complemented an ATR-deficient bacterial strain. These authors demonstrated decreased expression of ATR by Western blot analysis in fibroblasts from three *cblB* patients [7].

The human *MMAB* gene product is a member of the PduO family of cobalamin adenosyltransferases. Recently, the crystal structure of an *MMAB* homologue from the archaean *Thermoplasma acidophilum* (gi|16082403, TA1434) has been determined to a resolution of 1.5 Å [8]. The enzyme was shown to function as a homotrimer, with a putative active site formed by interaction of adjacent subunits. Incorporation of two mutations described in human *cblB* patients into the *T. acidophilum* enzyme was shown to abolish adenosyltransferase activity in vitro. In addition to catalyzing adenosylation of cob(I)alamin, the *MMAB* gene product may also function as a chaperone, delivering AdoCbl in an activated state to MCM [9–11].

In the present study, we have sequenced the *MMAB* gene and surrounding intronic sequences in genomic DNA of 35 *cblB* patients.

Materials and methods

Patients

Cultured fibroblasts from patients suspected of having an inborn error of cobalamin metabolism have been sent to our laboratory for clinical diagnosis and characterization for the past 22 years. Cell lines were assigned a unique identification code by The Repository for Mutant Human Cell Strains of the Montreal Children's Hospital (http://www.cellbank.mcgill.ca), and these codes were subsequently used. The referring physicians indicated whether cell lines should not be used to pursue research into the origin of these diseases. If physicians were contacted to request samples of DNA or cell lines from additional family members, informed consent was obtained from the patient or his/her family. This research protocol was approved by the Royal Victoria Hospital Research Ethics Board.

Studies were carried out on fibroblasts from 35 *cblB* patients (19 males; 14 females; two not specified). This panel included six cell lines (WG1185, WG2027, WG2186, WG2492, WG2523, and WG2633) previously sequenced by Dobson et al. [6], as well as three lines (WG1680, WG1879, and WG2127) studied by Leal et al. [7]. Results of cell culture studies of these fibroblast lines are shown in Table 1. The majority of these patients were reported to be of European ancestry with a subset of patients of Middle Eastern (six patients) and African (five patients) ancestry. Three patients were reported as offspring of consanguinous unions (one first cousin, one second cousin, and one not specified). Only unrelated patients were included in mutation analyses. Patient fibroblast lines had decreased incorporation of label from [^{14}C]propionate into cellular macromolecules (a measure of MCM function in intact cells) and, in most cases, decreased synthesis of AdoCbl from exogenous [^{57}Co]CNCbl; in two patient fibroblast lines (WG1771 and WG2027), synthesis of AdoCbl fell within the control range. In all cases, the diagnosis of *cblB* was established by somatic cell complementation analysis [12,13].

DNA sequencing

Sequencing of the *MMAB* gene (NM_052845) was carried out on 35 *cblB* patients and 50 unrelated CEPH controls. DNA was extracted using the Qiagen genomic DNA extraction kit (Mississauga, Ontario, Canada) for cultured cells. *MMAB* exons 1–9 and flanking sequences were amplified by polymerase chain reaction (PCR) using primers described by Dobson et al. [6]. PCR products were purified with Montage PCR96 filter plates (Millipore, Bedford, MA). Purified PCR products were used for 10 μL sequencing reactions made up of 2 μL PCR product, 1 μL of BigDye Terminator Cycle Sequencing Version 3.1 (Applied Biosystems, Foster City, CA), 1.5 μL of 5× buffer, 5 μL H_2O, and 0.5 μL [1 μM final] of sense or antisense primer. Amplicons were sequenced in both forward and reverse directions. Products were analyzed on an ABI3730 Sequence Analyzer (Applied Biosystems). Sequencing files were processed using Sequence Analysis software (PE Applied Biosystems) and were assembled and analyzed using the Phred/Phrap/Consed System [14,15]. DNA mutation numbering is based on cDNA sequence with +1 corresponding to the A of the ATG translation initiation codon.

Haplotypes were determined in homozygous *cblB* patients using 10 single nucleotide polymorphisms (SNPs) within the *MMAB* gene that were sequenced during mutation analysis. The SNPs used were (from 5′ to 3′): rs10774774 (exon 1), rs10774775 (exon 1), rs2287180 (exon 7), rs12309115 (exon 7), rs2287181 (exon 7), rs11610545 (exon 7), rs2287183 (exon 8), rs2287182 (exon 8), rs8228 (exon 9), and rs9593 (exon 9) (http://www.ncbi.nih.gov/snp/). Frequency of haplotypes in control CEU samples (CEPH, Utah) were determined from the haplotype map developed by the International HapMap consortium using the program Haploview [16,17].

Results

Results of mutation analysis in the panel of 35 *cblB* fibroblast lines are shown in Table 1. At least two mutations were identified in 34 patients; in the remaining patient, only a single mutation was identified. Three apparent mutations were identified in two patients (WG2487 and WG2492). Nineteen different *MMAB* mutations were observed in our panel. These include 11 missense mutations, one nonsense mutation, one duplication causing a frameshift, one duplication without a frameshift, one deletion causing a frameshift, and four mutations affecting splice sites. Of these mutations, 13 have not been previously described. In addition, several previously described mutations were observed in additional patients. Over half of the mutations (11/19) were localized to exon 7 (Fig. 1).

Table 1
Mutation and biochemical analysis of patients with *cblB*

Patient cell line	Sex/race	Age of onset	Mutation 1		Mutation 2		Prop − OH	Prop + OH	AdoCbl
			cDNA	Protein	cDNA	Protein			
WG2816	Unknown	<23 days	c.197-1G > T	r.spl?	c.197-1G > T	r.spl?	0.4	0.4	3.8
WG3176	M/W	3.8 years	c.197-1G > T	r.spl?	c.197-1G > T	r.spl?	0.5	0.4	ND
WG3293	M/W	14 years	c.197-1G > T	r.spl?	c.197-1G > T	r.spl?	0.6	0.7	4.1
WG2523	F/W	7 months	c.197-1G > T	r.spl?	c.577G > A	p.E193K	0.2	0.5	1.2
WG3185	M/W	11 months	c.290G > A	p.G97E	c.568C > T	p.R190C	1.1	2.1	6.1
WG2350	M/W	3 days	c.291-1G > A	r.spl?	c.291-1G > A	r.spl?	0.5	0.5	1.6
WG2027[a]	M/W	3 days	c.291-1G > A	r.spl?	c.556C > T	p.R186W	0.9	0.9	13.1
WG3332	F/B/W	4 days	c.291-1G > A	r.spl?	c.700C > T	p.Q234X	1.7	5.2	1.6
WG2235	F/W	12 years	c.521C > T	p.S174L	c.521C > T	p.S174L	4.4	5.4	3.6
WG2980	M/A	14 days	c.539C > G	p.S180W	c.568C > T	p.R190C	0.5	1.0	1.0
WG1185	Unknown	Unknown	c.556C > T	p.R186W	c.556C > T	p.R186W	1.1	ND	4.3
WG1493	M/W	3 days	c.556C > T	p.R186W	c.556C > T	p.R186W	0.3	0.4	2.2
WG2186	M/W	<1 week	c.556C > T	p.R186W	c.556C > T	p.R186W	1.0	1.1	2.5
WG1771[a]	M/W	3 weeks	c.556C > T	p.R186W	c.556C > T	p.R186W	1.8	2.0	9.7
WG1792	M/W	3 months	c.556C > T	p.R186W	c.556C > T	p.R186W	3.4	4.1	2.1
WG1586	F/W	7 months	c.556C > T	p.R186W	c.556C > T	p.R186W	1.1	1.3	4.0
WG2846	F/W	11 months	c.556C > T	p.R186W	c.556C > T	p.R186W	0.8	1.0	1.1
WG3117	F/W	18 months	c.556C > T	p.R186W	c.556C > T	p.R186W	0.9	1.2	2.1
WG1879	M/W	5 months	c.556C > T	p.R186W	c.569G > A	p.R190H	1.0	1.5	3.4
WG2779	F/W	21 months	c.556C > T	p.R186W	c.572G > A	p.R191Q	1.2	2.3	2.5
WG2633[b]	F/W	7 days	c.556C > T	p.R186W	c.563_577dup	p.186_190dup	0.5	0.5	0.8
WG2268	M/W	2 days	c.556C > T	p.R186W	c.585-2A > C	r.spl?	0.7	0.6	3.3
WG2345	M/W	1 year	c.556C > T	p.R186W	c.700C > T	p.Q234X	2.8	5.2	6.0
WG2776	F/W	6.5 years	c.556C > T	p.R186W	c.700C > T	p.Q234X	2.8	4.2	2.3
WG3274	M/W	14 years	c.557G > A	p.R186Q	c.557G > A	p.R186Q	0.7	0.9	2.0
WG2147	M/B	5 days	c.563_577dup	p.186_190dup	c.563_577dup	p.186_190dup	0.8	1.0	3.3
WG3224[c]	F/W		c.567_571dup	p.R191PfsX25	c.567_571dup	p.R191PfsX25	0.5	0.4	1.3
WG3296	F/W	4 days	c.569G > A	p.R190H	c.569G > A	p.R190H	0.8	1.0	2.1
WG2492[d]	M/B	3 years	c.571C > T	p.R191W	c.656A > G	p.Y219C	2.0	3.6	6.5
WG2487[d]	F/B	8 years	c.571C > T	p.R191W	c.656A > G	p.Y219C	1.4	2.5	3.5
WG3230	M/B	3 days	c.585-2A > C	r.spl?	c.585-2A > C	r.spl?	0.9	0.7	0.4
WG2127	F/W	4 days	c.656_659del	p.Y219SfsX4	c.700C > T	p.Q234X	2.1	4.1	2.3
WG1641	M/W	Infancy	c.700C > T	p.Q234X	c.700C > T	p.Q234X	2.8	4.8	4.2
WG0117	F/unknown	11 months	c.700C > T	p.Q234X	c.700C > T	p.Q234X	2.4	ND	ND
WG1680[e]	M/W	3 days	c.700C > T	p.Q234X			2.8	5.0	2.8
cblB Avg[f]							1.4 ± 1.0	2.0 ± 1.7	3.4 ± 2.6
Controls[f] ($n = 12$)							10.8 ± 3.7	10.9 ± 3.5	15.3 ± 6.7

Cell lines are arranged sequentially by the position of the first mutation from 5′ to 3′, and then by the age of onset. F = female; M = male; W = white; B = black; A = Asian. ND = not done; r.spl? = mutation predicted to affect splicing. Prop-OH: incorporation of [^{14}C]propionate (nmol/mg prot/18 h) without addition of OHCbl. Prop + OH: incorporation of [^{14}C]propionate (nmol/mg prot/18 h) with addition of OHCbl. AdoCbl: AdoCbl synthesis (% of total intracellular Cbl).

[a] AdoCbl synthesis was within the control range in two patient fibroblast lines (WG1771 and WG2027); diagnosis of *cblB* in these patients was established by somatic cell complementation analysis.

[b] The previously reported c.572_576delGGGCC mutation could not be detected in this cell line [6].

[c] Methylmalonic aciduria was detected in the newborn period; patient was investigated after birth because of previously affected siblings.

[d] Three sequence changes detected; third sequence change (c.403G > A [A135T]) not shown.

[e] A single mutation (c.700C > T) was detected in heterozygous form in WG1680; the second mutation remains unknown.

[f] Values for *cblB* average and controls are means ± SD.

Novel mutations

The 13 novel mutations included seven missense mutations as well as one nonsense mutation, two duplications, one deletion, and two splice mutations. None of these sequence changes was identified in 100 control alleles. Two duplications (c.567_571dupCCGCC and c.563_577dupTGTGCCGCCG GGCCG) were identified in exon 7. Of these, c.567_571dupCCGCC (p.R191PfsX25) results in a frame shift and predicts a truncated protein product lacking part of the putative enzyme active site. The in-frame insertion c.563_577dupTGTGCCGCCGGGCCG (p.186_ 190dup) would result in the insertion of five additional amino acids within the active site. The c.585-2A > C mutation results in disruption of the splice acceptor site at the beginning of exon 8 and predicts an improperly spliced mRNA. The c.290G > A (p.G97E) mutation alters the final base of exon 3, part of the consensus splice site, and is predicted to affect proper splicing of the *MMAB* transcript. In addition, it would result in the replacement of a glycine residue at position 97 of the protein by glutamic acid; this glycine is conserved in PduO cobalamin adenosyltransferases from eukaryotes, bacteria, and archaea.

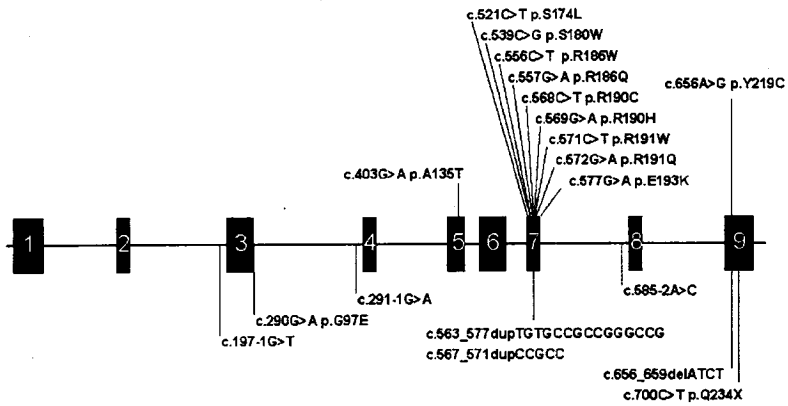


Fig. 1. Mutations identified in the *MMAB* gene. The gene structure of the *MMAB* gene according to reference sequence NM_052845.2 is shown. Missense mutations are shown above the plane of the gene; duplications, deletions, splice-site mutations, and a premature termination codon mutation are shown below the plane of the gene.

The c.700C>T (p.Q234X) mutation results in the creation of a stop codon at position 234 and is predicted to result in instability of the message or truncation of the protein product.

The remaining novel mutations were missense mutations. Four of these (c.557G>A [p.R186Q], c.568C>T [p.R190C], c.569G>A [p.R190H], and c.572G>A [p.R191Q]) affected invariant arginine residues within the putative active site [8] and would be predicted to affect enzyme activity. Specifically, multiple amino acid substitutions at Arg191 have been shown experimentally to decrease or eliminate enzyme activity in the *T. acidophilum* adenosyltransferase in vitro [8]. The c.521C>T (p.S174L) and c.539C>G (p.S180W) mutations result in the replacement of residues in the region of the enzyme active site. While neither residue was identified as part of the active site [8], both were highly conserved in the analysis of 22 adenosyltransferases from eukaryotes, bacteria, and archaea [7].

Three different sequence changes (c.403G>A [p.A135T], c.571C>T [p.R191W], and c.656A>G [p.Y219C]) were observed in two patients in our panel. Two of these—c.403G>A (p.A135T) and c.571C>T (p.R191W)—were previously reported as mutations in WG2492 [6]. p.R191W abolishes adenosyltransferase activity in the *T. acidophilum* enzyme in vitro [8]. Of the other two mutations, p.Y219C affects a well-conserved residue adjacent to three invariant residues identified as part of the active site (Glu214, Arg215, and Ser217), and the presence of cysteine with its thiol group within the enzyme active site might be expected to deleteriously affect activity. The p.A135T change affects a residue that is poorly conserved [7,8].

Common mutations

The previously reported c.197-1G>T, c.291-1G>A, c.403G>A (p.A135T), c.556C>T (p.R186W), and c.571C>T (p.R191W) mutations [6] were observed in additional patients in our study. Overall, the c.556C>T (p.R186W) mutation was the most frequent in our panel of patients, accounting for 23 alleles (33% of all alleles in the panel). Other frequently observed alleles were c.700C>T (p.Q234X) (nine alleles), c.197-1G>T (seven alleles), and c.291-1G>A (four alleles).

Among the common mutations, c.556C>T (p.R186W) was seen almost exclusively among patients of European descent (one homozygous patient was described as Mexican). All patients homozygous for the c.556C>T mutation shared a common haplotype constructed from 10 SNPs identified within the *MMAB* gene; however, the same haplotype was also associated with other mutations (c.197-1G>T, c.291-1G>A, and c.563_577dupTGTGCCGCCG GGCCG) in patients of various ethnic backgrounds. Two SNPs that were typed in these patients (rs2287182 and rs9593) were also typed by the HapMap Consortium (v.19). The haplotype associated with the c.556C>T mutation was the second most frequent haplotype observed in the CEU samples (CEPH, Utah), accounting for 13% of chromosomes in this population [16].

The c.197-1G>T splice-site mutation was seen in heterozygous or homozygous form in three Saudi *cblB* patients; the ethnicity of the fourth *cblB* patient carrying this mutation was not specified. The c.403G>A (p.A135T), c.571C>T (p.R191W), and c.656A>G (p.Y219C) mutations were seen together in two African-American patients; no other patients had any of these mutations.

Genotype and phenotype correlation

The majority of *cblB* patients in our collection came to medical attention within the first year of life, but clinical presentation ranged from the neonatal period to 14 years of age (Table 1). In addition, age at presentation was an incomplete reflection of disease severity; one patient in our panel died following a first acidotic crisis at the age of 12 years. The common c.556C>T mutation was usually associated with early onset; there were seven patients with known clinical course who were homozygous for c.556C>T, with age at presentation ranging from the neonatal period to 18 months

Table 2
Effect of genotype on biochemical parameters in *cblB* patients

Genotype	Propionate incorporation (nmol/mg prot/18 h)		AdoCbl (% of total)
	Without OHCbl	With OHCbl	
cblB ($n = 35$)	1.4 ± 1.0 (0.2–4.4)	2.0 ± 1.7 (0.4–5.4)	3.4 ± 2.6 (0.4–13.1)
c.556C > T/c.556C > T ($n = 8$)	1.3 ± 0.9 (0.3–3.4)	1.6 ± 1.2 (0.4–4.1)	3.5 ± 2.6 (1.1–9.7)
c.197-1G > T/c.197-1G > T ($n = 3$)	0.4; 0.5; 0.6	0.4; 0.4; 0.7	3.8; ND; 4.1
c.700C > T/c.700C > T ($n = 2$)	2.8; 2.4	4.8; ND	4.2; ND
c.700C > T/c.556C > T ($n = 2$)	2.8; 2.8	5.2; 4.2	6.0; 2.3
c.700C > T/other[a] ($n = 6$)	2.5 ± 0.5 (1.7–2.8)	4.8 ± 0.5 (4.2–5.2)	3.2 ± 1.6 (1.6–6.0)

Values are expressed as means ± SD, with range in parentheses; when the number of patients was three or less, individual values are given.
ND = not done.
[a] Compound heterozygous patients carrying c.700C > T plus any other mutation.

(all but one presented in the first year). The c.197-1G > T mutation was seen in homozygous form in three patients; onset was at 3.8 and 14 years in two Saudi patients, but within the first month of life in a patient from North America. Both the African-American *cblB* patients with the c.403G > A, c.571C > T, and c.656A > G mutations had late presentation (3 years and 8 years). The affected sister of one of these patients (not part of this panel) also carried the same mutations and presented at 14 years of age.

The effect of some of the most common mutations on biochemical function of fibroblasts was assessed. Incorporation of label from [^{14}C]propionate into cellular macromolecules, which is a measure of intact cell function of MCM, tended to be decreased in cell lines homozygous for the c.197-1G > T mutation compared to other *cblB* lines (Table 2); there was no response of propionate incorporation to supplementation of culture medium with OHCbl. Fibroblasts from cell lines carrying the c.700C > T (p.Q234X) mutation in either homozygous or heterozygous form had relatively high propionate incorporation with a vigorous response to supplementation with OHCbl. There were no obvious correlations between specific mutations and AdoCbl synthesis in our assay system.

Discussion

In the present study, sequence analysis of genomic DNA from a panel of *cblB* patient fibroblasts has been used to identify disease-causing mutations in the *MMAB* gene. Thirteen novel mutations were identified, bringing the total number of *MMAB* mutations identified in our laboratory to 19. Three additional mutations, c.558_559delGGinsC [18], c.287T > C (p.I96T), and c.584G > A (affecting a splice site) [19,20] have been reported. Of particular note, the previously described c.556C > T (p.R186W) mutation represented 33% of all disease-causing alleles in our collection of 35 patients. This mutation has also been reported by other researchers [18–20]. In homozygous form, it was associated with onset of symptoms typically within the first year of life; biochemical studies in patient fibroblasts were typical of the *cblB* class as a whole (Table 2). Patients carrying this mutation were almost exclusively of European background and shared a common haplotype across the *MMAB* gene, consistent with a single mutational event. However, this haplotype was observed frequently in controls and was also observed in patients with other *MMAB* mutations, from other ethnic backgrounds. It is possible that the frequency of the c.556C > T mutation (a C to T transition at a CpG) is the result of more than one mutational event occurring on a common haplotype.

The recent determination of the crystal structure of a PduO class cobalamin adenosyltransferase from *T. acidophilum*, which is 32% identical to the human *MMAB* gene product, has provided insight into the structure of the active site of the enzyme [8]. The archaean enzyme functions as a homotrimer, with an active site formed by interaction of helix α1 of one subunit with the α4 helix of the adjacent subunit. A number of highly conserved residues located at the interface of two subunits, which are not involved in amino acid packing, were identified; it was suggested that these residues are involved in the formation of the active site. A number of the *MMAB* mutations identified in this study (c.556C > T [p.R186W], c.557G > A [p.R186Q], c.568C > T [p.R190C], c.569G > A [p.R190H], c.571C > T [p.R191W], c.572G > A [p.R191Q], and c.577G > A [p.E193K]) alter one of these highly conserved residues. Two of these mutations (p.R186W and p.E193K) have been shown to eliminate the activity of the *T. acidophilum* adenosyltransferase in vitro [8]. The remaining missense mutations identified (c.521C > T [p.S174L], c.539C > G [p.S180W], and c.656A > G [p.Y219C]) also affect conserved residues within or close to the enzyme active site. Verification that these mutations affect enzyme function will require additional in vitro enzymological studies.

A single nonsense mutation (c.700C > T [p.Q234X]) was identified. This mutation occurs close to the 3′-end of the gene and does not affect any conserved portion of the protein. This mutation may affect stability of mRNA or of the protein product. Fibroblasts from patients with this mutation in homozygous or heterozygous form had higher levels of MCM function than most *cblB* fibroblasts (Table 2), although this does not appear to be correlated with any reduction in severity of clinical presentation.

The remaining mutations included mutations predicted to affect splicing of mRNA and duplications or deletions.

Of note, the c.290G>A mutation is predicted to affect splicing between exons 3 and 4, and is also predicted to result in an amino acid change, p.G97E, affecting a conserved residue close to part of the active site of the protein product.

In two African-American *cblB* patients (WG2487 and WG2492), the same three sequence changes were detected within the *MMAB* gene. One change, c.571C>T (p.R191W), has been shown to abolish enzyme activity in the *T. acidophilum* adenosyltransferase. Of the remaining mutations, c.656A>G (p.Y219C) represents a nonconservative alteration at a conserved residue and may be a better candidate for a second disease-causing mutation than the c.403G>A (p.A135T) mutation, which was previously identified as a mutation in WG2492 [6]. Presumably, two of the sequence changes exist in cis in these patients.

Eleven of the 19 mutations identified in this study are in exon 7 of the *MMAB* gene. These include two duplication mutations (c.563_577dupTGTGCCGCCGGGCCG and c.567_571dupCCGCC); this may reflect the presence of a GC-rich region that includes repetitive elements. Four of the nine missense mutations identified in exon 7 represent C to T transitions at CpG sites (c.521C>T, c.556C>T, c.568C>T, and c.571C>T); cytosine residues at such sites have been recognized as particularly prone to mutation [21].

It was possible to identify some correlations between *MMAB* genotype and phenotype, both in clinical presentation and in behavior of cultured fibroblasts. However, there does not appear to be any consistent correlation between the cellular and the clinical phenotypes in *cblB* patients. Thus, the c.700C>T (p.Q234X) mutation in homozygous or heterozygous form was associated with a relatively mild defect in propionate incorporation in cultured fibroblasts, and a vigorous response of propionate incorporation to exogenous OHCbl (Table 2). However, this was not reflected in later age of onset of disease symptoms in patients carrying this mutation (Table 1). Similarly, the c.197-1G>T mutation was associated with a severe cellular phenotype, but patients homozygous for this mutation had relatively late ages at onset.

It has been noted that the *cblB* disorder generally has a more severe clinical course and is less responsive to therapy than the *cblA* disorder, although both disorders result in decreased AdoCbl synthesis. This has been attributed to the relative "leakiness" of *cblA* mutations [1]. It is notable that nearly all of the missense mutations that have been identified in *cblB* patients affect the putative active site of the adenosyltransferase. Thus, nearly all of the mutations identified in our collection are predicted to either cause formation of a truncated protein product (duplications and deletions, splice-site mutations) or of a protein with a defective active site. This may reflect in part the high GC content and repetitive nature of exon 7, which encodes part of the enzyme active site. It is also possible that missense mutations affecting amino acid residues that occur outside of the active site result in a phenotype that is not recognized clinically as the *cblB* disorder; this might account for a portion of the patients with relatively mild methylmalonic aciduria that cannot currently be assigned to any of the known inborn errors of cobalamin metabolism.

Acknowledgments

We thank the clinicians who provided patient samples and clinical information, Nora Matiaszuk for complementation analysis, Gail Dunbar for growth of patient fibroblasts, and Vince Forgetta, Donna Sinnett, Corine Zotti, and David Roquis for technical assistance. This is a publication of the Hess B. and Diane Finestone Laboratory in the memory of Jacob and Jenny Finestone.

References

[1] D.S. Rosenblatt, D.W.A. Fenton, Inherited disorders of folate and cobalamin transport and metabolism, in: C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle (Eds.), The Metabolic and Molecular Bases of Inherited Disease, vol. III, McGraw-Hill, New York, 2001, pp. 3897–3933.

[2] S.M. Matsui, M.J. Mahoney, L.E. Rosenberg, The natural history of the inherited methylmalonic acidurias, N. Engl. J. Med. 308 (1983) 857–861.

[3] C.M. Dobson, T. Wai, D. Leclerc, H. Kadir, M. Narang, J.P. Lerner-Ellis, T.J. Hudson, D.S. Rosenblatt, R.A. Gravel, Identification of the gene responsible for the *cblB* complementation group of vitamin B_{12}-dependent methylmalonic aciduria, Hum. Mol. Genet. 11 (2002) 3361–3369.

[4] W.A. Fenton, L.E. Rosenberg, Genetic and biochemical analysis of human cobalamin mutants in cell culture, Annu. Rev. Genet. 12 (1978) 223–248.

[5] N. Korotkova, M.E. Lidstrom, MeaB is a component of the methylmalomyl-CoA mutase complex required for protection of the enzyme from inactivation, J. Biol. Chem. 279 (2004) 13652–13658.

[6] C.M. Dobson, T. Wai, D. Leclerc, A. Wilson, X. Wu, C. Doré, T.J. Hudson, D.S. Rosenblatt, R.A. Gravel, Identification of the gene responsible for the *cblA* complementation group of vitamin B_{12}-responsive methylmalonic acidemia based on analysis of prokaryotic gene arrangements, Proc. Natl. Acad. Sci. USA 99 (2002) 15554–15559.

[7] N.A. Leal, S.D. Park, P.E. Kima, T. Bobik, Identification of the human and bovine ATP:Cob(I)alamin adenosyltransferase cDNAs based on complementation of a bacterial mutant, J. Biol. Chem. 278 (2003) 9227–9234.

[8] V. Saridakis, A. Yakunin, X. Xu, P. Anandakumar, M. Pennycooke, J. Gu, F. Cheung, J.M. Lew, R. Sanishvili, A. Joachimiak, C.H. Arrowsmith, D. Christendat, A.M. Edwards, The structural basis for methylmalonic aciduria. The crystal structure of archaeal ATP:cobalamin adenosyltransferase, J. Biol. Chem. 279 (2004) 23646–23653.

[9] M. Yamanishi, T. Labunska, R. Banerjee, Mirror "base-off" conformation of coenzyme B_{12}in human adenosyltransferase and its downstream target, methylmalonyl-CoA mutase, J. Am. Chem. Soc. 127 (2005) 526–527.

[10] T.A. Stich, M. Yamanishi, R. Banerjee, T.C. Brunold, Spectroscopic evidence for the formation of a four-coordinate Co^{2+}cobalamin species upon binding to the human ATP:cobalamin adenosyltransferase, J. Am. Chem. Soc. 127 (2005) 7660–7661.

[11] M. Yamanishi, M. Vlasie, R. Banerjee, Adenosyltransferase: an enzyme and an escort for coenzyme B12? Trends Biochem. Sci. 30 (2005) 304–308.

[12] D. Watkins, Cobalamin metabolism in methionine-dependent human tumour and leukemia cell lines, Clin. Invest. Med. 21 (1998) 151–158.

[13] D. Watkins, N. Matiaszuk, D.S. Rosenblatt, Complementation studies in the cblA class of inborn error of cobalamin metabolism: evi-

dence for interallelic complementation and for a new complementation class (cblH), J. Med. Genet. 37 (2000) 510–513.
[14] B. Ewing, L. Hillier, M.C. Wendl, P. Green, Base-calling of automated sequencer traces using Phred I. Accuracy assessment, Genome Res. 8 (1998) 175–185.
[15] D. Gordon, C. Abajian, P. Green, Consed: a graphical tool for sequence finishing, Genome Res. 8 (1998) 195–202.
[16] The International HapMap Consortium, A haplotype map of the human genome. Nature 437 (2005) 1299–1320.
[17] J.C. Barrett, B. Fry, J. Maller, M.J. Daly, Haploview: analysis and visualization of LD and haplotype maps, Bioinformatics 21 (2005) 263–265.
[18] E. Pospisilova, L. Mrazova, D. Priby, P. Chrastina, R. Soucek, O. Martincova, S. Stastna, K. Halova, D. Kantarska, E. Gregova, M. Kosarova, I. Chodorova, H. Dvorakova, M. Elleder, J. Zeman, Prenatal diagnosis in two families with methylmalonic acidemia due to cobalamin B deficiency, J. Inherit. Metab. Dis. 28 (2005) 84.
[19] M.A. Martinez, A. Rincón, L.R. Desviat, B. Merinero, M. Ugarte, B. Pérez, Genetic analysis of three genes causing isolated methylmalonic acidemia: identification of 21 novel allelic variants, Mol. Genet. Metab. 84 (2005) 317–325.
[20] A. Rincón, B. Perez, B. Merinero, L.R. Desviat, M. Ugarte, Clinical, biochemical and genetic analysis of five methylmalonic acidemia (MMA) patients belonging to the cblB complementation group, J. Inherit. Metab. Dis. 28 (2005) 82.
[21] A.R. Templeton, A.G. Clark, K.M. Weiss, D.A. Nickerson, E. Boerwinkle, C.F. Sing, Recombinational and mutational hotspots within the human lipoprotein lipase gene, Am. J. Hum. Genet. 66 (2000) 69–83.

Available online at www.sciencedirect.com
SCIENCE DIRECT
Molecular Genetics and Metabolism 88 (2006) 315–321
Molecular Genetics and Metabolism
www.elsevier.com/locate/ymgme

Combined methylmalonic aciduria and homocystinuria (*cblC*): Phenotype–genotype correlations and ethnic-specific observations

Chantal F. Morel, Jordan P. Lerner-Ellis, David S. Rosenblatt *

Department of Human Genetics and Division of Medical Genetics, Department of Medicine, McGill University, Montreal, Que., Canada

Received 3 April 2006; accepted 3 April 2006
Available online 22 May 2006

Abstract

Methylmalonic aciduria and homocystinuria, *cblC* type (MIM 277400), is the most frequent inborn error of vitamin B_{12} (cobalamin, Cbl) metabolism, caused by an inability of the cell to convert Cbl to both of its active forms (MeCbl, AdoCbl). Although considered a disease of infancy, some patients develop symptoms in childhood, adolescence, or adulthood. The gene responsible for *cblC*, *MMACHC*, was recently identified. We studied phenotype–genotype correlations in 37 patients from published case-reports, representing most of the landmark descriptions of this disease. 25/37 had early-onset disease, presenting in the first 6 months of life: 17/25 were found to be either homozygous for the c.271dupA mutation ($n=9$) or for the c.331C>T mutation ($n=3$), or compound heterozygotes for these 2 mutations ($n=5$). 9/12 late-onset cases presented with acute neurological symptoms: 4/9 were homozygous for the c.394C>T mutation, 2/9 were compound heterozygotes for the c.271dupA and c.394C>T mutations, and 3/9, for the c.271dupA mutation and a missense mutation. Several observations on ethnic origins were noted: the c.331C>T mutation is seen in Cajun and French-Canadian patients and the c.394C>T mutation is common in the Asiatic-Indian/Pakistani/Middle Eastern populations. The recognition of phenotype–genotype correlations and the association of mutations with specific ethnicities will be useful for identification of disease-causing mutations in *cblC* patients, for carrier detection and prenatal diagnosis in families where mutations are known, and in setting up initial screening programs in molecular diagnostic laboratories. Further study into disease mechanism of specific mutations will help to understand phenotypic presentations and the overall pathogenesis in *cblC* patients.

Keywords: *cblC*; Methylmalonic aciduria; Homocystinuria; *MMACHC*; Genotype; Phenotype; Vitamin B12; Cobalamin

Introduction

In 1969, McCully proposed that hyperhomocysteinemia plays a role in the pathogenesis of arteriosclerosis [1]. This hypothesis was elaborated based on the pathological evidence of similar arterial damage seen in two patients with different inborn errors of metabolism both presenting with hyperhomocysteinemia: one "classic" homocystinuria, (cystathionine synthase deficiency, MIM 236200), and a second with a previously undescribed abnormality of cobalamin metabolism resulting in homocystinuria, cystathioninuria, and methylmalonic aciduria. The second patient was in fact the very first *cblC* patient described in the literature [2,3]. *cblC* (MIM 277400) is the most common inborn error of cobalamin metabolism, with over 300 patients diagnosed.

Vitamin B_{12} (cobalamin, Cbl), in the form of the cofactors methylcobalamin (MeCbl) and adenosylcobalamin (AdoCbl), is required by the cytoplasmic enzyme methionine synthase and by the mitochondrial enzyme methylmalonyl-CoA mutase, respectively. Inherited disorders of intracellular cobalamin metabolism are rare conditions, and have been classified on the basis of somatic cell complementation as belonging to eight different mutant groups (*cblA* to *cblH*) [4]. Functional deficiencies of methionine synthase lead to homocystinuria and elevated total plasma homocysteine (*cblE*, *cblG*, and *cblD* variant 1), while a primary defect in mutase or in the synthesis of AdoCbl lead to methylmalonic aciduria (*cblA*, *cblB*, *cblD* variant 2, and *cblH*). A deficiency of the

* Corresponding author. Fax: +1 514 934 8536.
E-mail address: david.rosenblatt@mcgill.ca (D.S. Rosenblatt).

1096-7192/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ymgme.2006.04.001

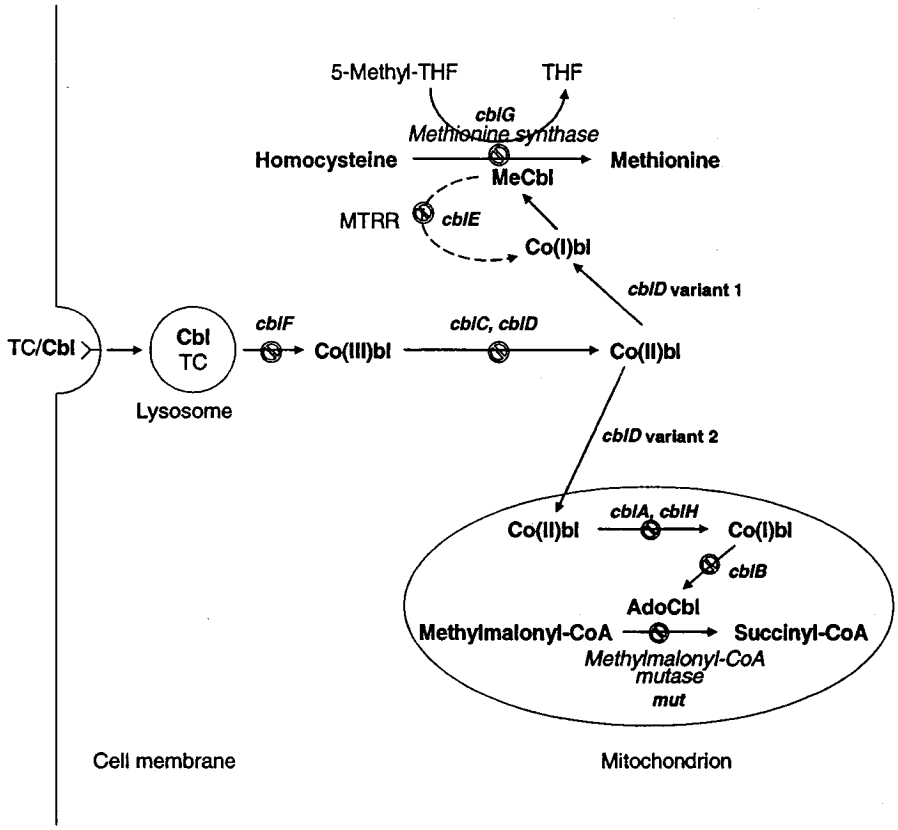


Fig. 1. Summary of inborn errors of Cbl metabolism, including TC deficiency and mutase deficiency. AdoCbl, adenosylcoblamin; Cbl, cobalamin, *cblA-cblG*, Cbl complementation group diseases; MeCbl, methylcobalamin; MTRR, methionine synthase reductase; Mut, methylmalonyl-CoA mutase deficiency; TC/Cbl, transcobalamin–cobalamin complex; TC, transcobalamin; THF, tetrahydrofolate.

enzymes involved in the early steps of cellular vitamin B_{12} metabolism lead to accumulation of both homocysteine and methylmalonic acid in blood and urine (*cblC*, *cblD*, and *cblF*). Fig. 1 summarizes these conditions.

cblC was originally thought to be primarily a disease of infancy, presenting with failure to thrive, acute neurological deterioration, mental retardation, retinopathy, multisystem organ dysfunction, and haematological abnormalities including megaloblastic anemia [4]. In 1984, Shinnar and Singer [5] reported a previously asymptomatic 14-years-old girl diagnosed with *cblC* who presented with an acute onset of neurological symptoms, including dementia, myelopathy, and motor neuron disease: this case-report was pivotal in establishing the heterogeneity of this condition. In 1997, Rosenblatt et al. [6] reviewed 50 *cblC* patients, of whom six patients had late-onset. The authors concluded that the late-onset patients had better survival and response to treatment, and less neurological sequelae compared to the early-onset patients. Various case-reports describing late-onset cases followed suite [7–14].

Recently, we identified the gene responsible for this condition by homozygosity mapping and haplotype analysis [15]. The gene was named *MMACHC* (for methylmalonic aciduria *cblC* type with homocystinuria): it is comprised of four coding exons and a 5th non-coding exon. Molecular modeling predicts that the C-terminal region of the gene product folds similarly to TonB, a bacterial protein involved in energy transduction for vitamin B_{12}-uptake.

Subjects and methods

Subjects

For the past 22-years cultured fibroblasts from patients suspected of having an inborn error of Cbl metabolism have been sent to our laboratory for clinical diagnosis and characterization. A unique identification code was assigned to cell lines by the Repository for Mutant Human Cell Strains at the Montreal Children's Hospital (http://www.cellbank.mcgill.ca). Referring physicians indicated whether cell lines should not be used to pursue research into the origin of these diseases. The procedure was approved by the Royal Victoria Hospital Research Ethics Board. Thirty-seven patients in this collection have been the subjects of previous publications [16,5,7,17–27,8,28,9–11,29,12,30,31,13,32,14]. The diagnosis of *cblC* was made based on Cbl distribution studies as per the methods outlined in Rosenblatt et al. [33], as well as incorporation of label from [^{14}C]propionate and 5-[^{14}C]methyltetrahydrofolate into cellular macromolecules and complementation studies as outlined by Watkins et al. [34]. This paper focuses on published case-reports of *cblC* patients, assigns causal mutations to many of the reported individuals and examines phenotypic correlations.

Mutation analysis

The open-reading frame and flanking exon sequences of the *MMACHC* gene, and the non-coding portion of exon 4 and exon 5, were sequenced

from genomic DNA. PCR products were purified with Montage PCR_{96} filter plates (Millipore). Sequencing reactions (10 μl) contained 2 μl purified PCR product, 1 μl BigDye Terminator Cycle Sequencing Version 3.1 (Applied Biosystems), 1.5 μl of 5× buffer, 5 μl H_2O, and 0.5 μl (1 μM final) of sense or antisense primer. Products were analyzed on an ABI 3730 × 1 DNA Analyzer (Applied Biosystems). Sequencing files were processed using Sequence Analysis software (Applied Biosystems) and were assembled and analyzed using the Phred/Phrap/Consed System [35,36]. We have recently reported 42 mutations identified in 204 patients [15].

Results

Mutation analysis

Out of the thirty-seven published cases, nine were homozygous for the c.271dupA mutation (patients 6, 7, 11, 12, 14, 17, 18, 27, and 36); this particular mutation has been reported to account for 40% of mutant alleles [15]. Patients 2, 3, 9, and 37 were homozygous for the c.394C>T (p.R132X) mutation. Patients 15, 16, and 20 were homozygous for the c.331C>T (p.R111X) mutation. Two patients (32 and 33) were homozygous for the c.440G>A (p.G147D) mutation. Three patients, 1, 4, and 19, were heterozygous for the c.271dupA and c.394C>T (p.R132X) mutations. Five patients, (5, 8, 21, 22, and 23), were heterozygous for c.271dupA and c.331C>T (p.R111X). Three patients were heterozygous for c.271dupA and c.82-9_12delTTTC (patients 26, 34, and 35). Table 1 summarizes the mutations identified in the thirty-seven patients included in this study.

Phenotype–genotype correlations

Twenty-five of the thirty-seven (25/37) had early-onset disease, presenting in the first 6 months of life. 17/25

Table 1
Clinical and molecular data on published patients, listed in order of year of publication

Pt	Reference	Sex	Onset	Ethnicity	Mutation 1	Mutation 2
1	Dillon et al.[16]	F	Early	Not disclosed	c.271dupA	c.394C>T
2[a]	Shinnar, Singer[5]	F	Late	Asiatic-Indian	c.394C>T	c.394C>T
3[a]		F	Asymp	Asiatic-Indian	c.394C>T	c.394C>T
4	Mitchell et al.[7]	M	Late	Portuguese	c.271dupA	c.394C>T
5		F	Early	French-Canadian	c.271dupA	c.331C>T
6	Bartholomew et al.[17]	M	Early	White	c.271dupA	c.271dupA
7		F	Early	White	c.271dupA	c.271dupA
8	Brandstetter et al.[18]	M	Early	White	c.271dupA	c.331C>T
9	Kazimiroff, Shaner[19]	F	Late	Asiatic-Indian	c.394C>T	c.394C>T
10	Wijburg et al.[20]	M	Early	Turkish[b]	c.80A>G	c.80A>G
11	Geraghty et al.[21]	F	Early	White	c.271dupA	c.271dupA
12	Russo et al.[22]	M	Early	French-Canadian	c.271dupA	c.271dupA
13	Howard et al.[23]	F	Early	Hispanic	c.331C>T	c.615C>G
14		F	Early	Hispanic	c.271dupA	c.271dupA
15	Andersson, Shapira[24]	M	Early	White-Louisiana	c.331C>T	c.331C>T
16		F	Early	Cajun	c.331C>T	c.331C>T
17	Ellaway et al.[25]	M	Early	Middle Eastern[b]	c.271dupA	c.271dupA
18	Merinero et al.[26]	F	Early	White-Spain	c.271dupA	c.271dupA
13	Enns et al.[27]	See details on patient 13 published by Howard et al.[23]				
19	Augoustides-S. et al.[8]	F	Late	Greek	c.271dupA	c.394C>T
15	Andersson et al.[28]	See details on patient 15 published by Andersson, Shapira[24]				
20		F	Early	Cajun	c.331C>T	c.331C>T
16		See details on patient 16 published by Andersson, Shapira[24]				
21		M	Early	White-Louisiana	c.271dupA	c.331C>T
22		M	Early	White-Louisiana	c.271dupA	c.331C>T
23		F	Early	White-Louisiana	c.271dupA	c.331C>T
24	Powers et al.[9]	M	Late	White	c.271dupA	c.440G>C
25	Bodamer et al.[10]	M	Late	Hispanic	c.271dupA	c.482G>A
26	Van Hove et al.[11]	M	Late	White	c.82-9_12delTTTC	c.271dupA
27	Harding et al.[29]	M	Early	Middle Eastern[b]	c.271dupA	c.271dupA
28		F	Early	Hispanic	c.328_331delAACC	c.328_331delAACC
29	Roze et al.[12]	F	Late	Middle Eastern	c.271dupA	c.347T>C
30	Francis et al.[30]	F	Early	Asian	c.457C>T	c.481C>T
31	Heidenreich et al.[31]	F	Early	Native American	c.328_331delAACC	c.394C>T
32		F	Early	Native American	c.440G>A	c.440G>A
33		M	Early	Native American	c.440G>A	c.440G>A
34[a]	Guigonis et al.[13]	F	Late	White – France	c.82-9_12delTTTC	c.271dupA
35[a]		F	Late	White – France	c.82-9_12delTTTC	c.271dupA
36	Tsina et al.[32]	F	Early	White	c.271dupA	c.271dupA
37	Boxer et al.[14]	M	Late	Middle Eastern[b]	c.394C>T	c.394C>T

Pt, Patient; Asymp, Asymptomtic.
[a] Siblings.
[b] Consanguinity.

Table 2
Mutations and predicted effect on protein or mRNA

DNA	Protein	Type of mutation
c.3G > A	p.Met1?	Initiation codon change
c.80A > G	p.Gln27Arg	Missense
c.82-9_12delTTTC	r.spl?	Splice site
c.271dupA	p.Arg91LysfsX14	Frameshift-insertion
c.328_331delAACC	p.Asn110AspfsX13	Frameshift-insertion
c.331C > T	p.Arg111X	Nonsense
c.347T > C	p.Leu116Pro	Missense
c.394C > T	p.Arg132X	Nonsense
c.440G > A	p.Gly147Asp	Missense
c.440G > C	p.Gly147Ala	Missense
c.457C > T	p.Arg153X	Nonsense
c.481C > T	p.Arg161X	Nonsense
c.482G > A	p.Arg161Gln	Missense
c.615C > G	p.Tyr205X	Nonsense

patients were found to be either homozygous for the c.271dupA mutation ($n=9$) or for the c.331C > T mutation ($n=3$), or compound heterozygotes for these two mutations ($n=5$). Most of the remaining early-onset patients were either compound heterozygotes for different nonsense mutations, or for a nonsense mutation and a frameshift mutation (see Table 2 for details on types of mutation).

Nine of twelve (9/12) late-onset cases presented with acute neurological symptoms: 4/9 were homozygous for the c.394C > T mutation. These individuals (patients 2, 3, 9, and 37) had clinical courses characterized by normal development and health until early adolescence or adulthood with sudden neurological deterioration (except for the asymptomatic 12-year-old girl identified once the diagnosis was established in her older sister). 2/9 patients with late-onset disease were compound heterozygotes for the c.271dupA and c.394C > T mutations. Interestingly, patient 1, who also was a compound heterozygote for the c.271dupA and c.394C > T, had early-onset disease. 3/9 patients with late-onset disease were compound heterozygotes for the c.271dupA mutation and a missense mutation. The remaining 3/12 patients with late-onset disease had mainly renal involvement in absence of neurological manifestations: all were heterozygous for the c.271dupA mutation and the c.82-9_12delTTTC mutation. This intronic mutation was not seen in any other individual from the database of 204 sequenced patients.

Observations on ethnic background

Out of the nine published cases homozygous for the c.271dupA mutation, 6 patients are white (of European or North American extraction), one is Hispanic, one Iranian, and one Middle Eastern. From the database, another 44 patients from all ethnic backgrounds were found to be homozygous for the c.271dupA mutation. Therefore, this mutation was seen in individuals of various backgrounds.

Homozygosity for the c.331C > T (p.R111X) mutation in the published cases was seen exclusively in three Cajun patients (patients 15, 16, and 20). In addition, three unpublished patients of French-Canadian background from the database were also found to be homozygous for this mutation. Compound heterozygosity for the c.331C > T and c.271dupA mutations was identified in 5 published patients: one said to be white from the USA (patient 8), one French-Canadian (patient 5), and three from LA, USA (patients 21, 22, and 23). From the database's unpublished patients, an additional five individuals of French-Canadian or Cajun background were found to be heterozygous for the c.271dupA and c.331C > T mutations.

The c.394C > T (p.R132X) mutation, primarily associated with late-onset disease, was noted in homozygous state amongst four published individuals of Asiatic-Indian, Pakistani, or Middle Eastern descent (patients 2, 3, 9, and 37). Homozygosity for the c.394C > T mutation was seen in an additional nine unpublished patients from the database, all of whom were either Asiatic-Indian, Pakistani, or Middle Eastern. This mutation is seen in heterozygous state along with c.271dupA in two individuals of European ancestry (patients 4 and 19) and one patient of undisclosed ethnic background. In the database, no individual of European ancestry was homozygous for the c.394C > T mutation.

Homozygosity for the c.440G > A (p.G147D) mutation was noted only amongst Native American affected individuals (Patients 32 and 33, and a third non-published case from the original data published by Lerner-Ellis et al.).

Discussion

cblC has been demonstrated to be a heterogeneous condition. Presentation can occur in the neonatal period with multisystemic organ failure in the context of an acute metabolic crisis, by insidious developmental delay without metabolic illness, as a neurological deterioration in a previously well child, adolescence or adult, or as isolated renal disease. With the identification of the *MMACHC* gene, it has become possible to establish genotype–phenotype correlations, as well as observe ethnic-related trends. While the function of the protein remains to be elucidated, the pathological genetic defect specific to each patient can in part explain the heterogeneity that has come to light since Mudd and Levy's original case report [2,3].

Differences in RNA stability or residual function of the protein product are predicted to cause, at least in part, differences in phenotypes [37]. In addition, other factors such as background individual genetic variation, environmental, and dietary exposures may also account for the variation in presentation in individuals carrying the exact same two mutations. Guigonis et al. [13] reported 2 sisters affected with *cblC* who presented with thrombotic microangiopathy, suggesting haemolytic uremic syndrome, in absence of neurological involvement. The younger of the two sisters had more severe renal disease, associated with renal failure: she was found to be heterozygous for the 3254T > C Factor H polymorphism, which was felt by the authors to explain her more aggressive renal involvement. In addition, the patient reported by Augoustides-Savvo-

poulou et al. [8], who was found to be heterozygous for c.271dupA and c.394C>T, had onset of the disease after being previously developmentally and neurologically normal: her older sister was reported to have had onset of seizures in the neonatal period, developmental delay, spastic paraparesis, and died at 13 years of age, undiagnosed. It is probable that this child was also affected with *cblC* and that heterozygosity for c.271dupA and c.394C>T in this family would have led to an early-onset case and a late-onset case, demonstrating significant intrafamilial phenotypic heterogeneity in the presence of identical mutations. Another possibility, albeit less likely, is that one of the parents of these girls is a compound heterozygote for both an early-onset mutation and the c.394C>T, and the other parent a carrier of the c.271dupA mutation. However, the parent with compound heterozygosity would be expected to be symptomatic: genotyping of the parents would aid in clarifying if this is a case of familial phenotypic heterogeneity or of familial genotypic heterogeneity. Familial genotypic heterogeneity has been reported in the case of PKU and mild benign hyperphenylalaninemia in the same family [38,39] and could theoretically explain familial variability of phenotype in other metabolic conditions. Some additional factors that help to explain intrafamilial variability may include "synergistic heterozygosity" for disease-associated alleles encoding other enzymes or proteins in the cobalamin pathway, *cis* polymorphisms affecting folding or residual activity of the mutated enzyme, *trans* acting factors' interactions with the mutated allele, and non-genetic factors such as diet [40–43].

The mutation c.271dupA accounts for 40% of mutant alleles in our collection of 204 unrelated patients [15]. Upon reviewing the phenotype of patients homozygous for this mutation, the nine published cases were found to invariably have an early-onset (usually within the first 4 months of life), usually with an acute metabolic decompensation. Multisystem organ involvement at the time of diagnosis was not unusual, and most had a progression of their disease despite institution of OHCbl intramuscular (IM) injections. A significant proportion died, and of those who survived the initial presentation, none were cognitively and developmentally normal.

Lerner-Ellis et al. noted that patients who are homozygous for the c.394C>T (p.R132X) generally present later in life. The four published individuals with this genotype, including the first late-onset patients reported by Shinnar and Singer, demonstrate this finding. Patient three was asymptomatic at the time of diagnosis, whereas the three other individuals were cognitively and developmentally normal prior to the development of psychiatric and neurological symptoms. In addition, all three symptomatic individuals were responsive to OHCbl IM injections, with complete (or near complete) reversal of the psychiatric and neurological manifestations.

Two patients of the 12 published cases with late-onset disease were found to be compound heterozygous for the c.271dupA (early-onset) and c.394C>T (late-onset) mutations. These individuals did not present with acute metabolic decompensation early in life: both presented earlier than the four patients homozygous for the c.394C>T mutation, and later than the nine patients homozygous for the c.271dupA mutation. After a period of normal development, both individuals manifested acute psychiatric and neurological symptoms. OHCbl therapy reversed most of their symptoms. Therefore, it appears that these compound heterozygotes can manifest a disease intermediate between the severe early-onset form associated with homozygosity for c.271dupA and the late-onset phenotype associated with homozygosity for c.394C>T. However, patient one, who was also found to be compound heterozygote for these two mutations (as was the older sibling of patient 19, who presented in infancy), presented with a clinical course indistinguishable for the patients homozygous for the c.271dupA mutation, indicating that interpretation of anticipated phenotype based on this genotype may be unreliable.

The presence of c.331C>T in individuals of French-Canadian and Cajun ancestry may reflect a case of founder effect and genetic drift. The appearance of this mutation in Cajun individuals may be explained by the Deportation of the Acadians by the British in 1755, where many headed south to settle in Louisiana.

Different mutations at the same nucleotide are noted to produce strikingly different phenotypes, based on the predicted amino acid change. An example of this is demonstrated by the c.440G>A and c.440G>C mutations. The former is expected to cause a glycine to aspartic acid change at codon 147, while the latter is expected to be associated with a glycine to alanine change at the same codon. The c.440G>C (p.G147A) mutation was seen in compound heterozygous state with the c.271dupA mutation, in a patient (patient 24) who presented at the age of 45 years after a normal earlier life, with an unremarkable past medical history. In contrast, the c.440G>A (p.G147D) mutation, in compound heterozygous state with the c.271dupA, was associated with an early onset (all in the first month of life), and severe systemic involvement in two unpublished patients from the database of 204 sequenced patients. Codon 147 is located in the middle of the putative Cbl binding domain of the protein. Therefore, the substitution glycine by aspartic acid (a bulky, charged amino acid), may greatly disrupt the catalytic ability or conformational state of the protein, resulting in severe early-onset disease. Conversely, replacing glycine for the second smallest, non-polar amino acid alanine theoretically results in a lesser disruption of the protein's integrity and leads to a milder disease manifestation.

Late-onset of disease was seen frequently in patients who are compound heterozygotes for the c.271dupA mutation and a missense mutation: two examples of this from the published cases are those of patient 24 (c.271dupA/c.440G>C, diagnosed at 45 years) and patient 25 (c.271dupA/c.482G>A, diagnosed at 20 years). In fact, adolescent and adult-onset cases were mostly seen in the

c.394C>T homozygotes (as discussed above) and in compound heterozygotes for the c.271dupA and a missense mutation.

The c.82-9_12delTTTC mutation (in heterozygous state with the c.271dupA mutation) was only seen in three patients (patients 26, 34, and 35) with the unique presentation of haemolytic uremic syndrome without neurological involvement. Patient 26's younger sibling was also similarly affected. The c.82-9_12delTTTC mutation is a presumed splice site defect. It is possible that this mutation abolishes a particular splice site necessary to generate an *MMACHC* isoform essential for maintenance of renal integrity. OHCbl injections greatly improved renal function in all 4 patients.

The recognition of apparent phenotype–genotype correlations and the association of mutations with specific ethnicities or populations of origin will be immediately useful for identification of disease-causing mutations in *cblC* patients, for carrier detection in families where mutations are known, and in setting up initial screening programs in molecular diagnostic labs. There are potential implications for newborn screening: mutation screening tests for the more common early-onset mutations (i.e., c.271dupA and c.331C>T) could be offered in those infants with a positive screen for methylmalonic acid, thus consisting of a second-tier level in identifying *cblC* pre-symptomatically or early in the course of the disease. Molecular prenatal diagnosis could also be offered to couples at high risk of having affected children.

These findings demonstrate the translation of genomics into the application of a diagnostic tool. Further study into disease mechanism of specific mutations will help to understand phenotypic presentations and the overall pathogenesis in *cblC* patients.

Acknowledgments

In memory of Jamie C. Tirone. The authors would like to thank the referring physicians. We also thank David Watkins, Maria Galvez, Nora Matiaszuk, Gail Dunbar, and Jocelyne Lavallée for technical assistance. Funds from the CIHR (MGP-15078), the March of Dimes Birth Defects Foundation (6-FY01-11) and the Garrod Association contributed to this research. D.S.R. is a principal investigator in the CIHR Group in Medical Genetics. This is a publication of the Hess B. and Diane Finestone Laboratory in Memory of Jacob and Jenny Finestone.

References

[1] K.S. McCully, Vascular pathology of homocystinemia: implications for the pathogenesis of arteriosclerosis, Am. J. Pathol. 56 (1969) 111–128.

[2] S.H. Mudd, H.L. Levy, R.H. Abeles, J.P. Jennedy Jr., A derangement in B12 metabolism leading to homocystinemia, cystathioninemia and methylmalonic aciduria, Biochem. Biophys. Res. Commun. 35 (1969) 121–126.

[3] H.L. Levy, S.H. Mudd, J.D. Schulman, P.M. Dreyfus, R.H. Abeles, A derangement in B12 metabolism associated with homocystinemia, cystathioninemia, hypomethioninemia and methylmalonic aciduria, Am. J. Med. 48 (1970) 390–397.

[4] D.S. Rosenblatt, W.A. Fenton, Inherited disorders of folate and cobalamin transport and metabolism, in: C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle, B. Childs, K.W. Kinzler (Eds.), The Metabolic & Molecular Bases of Inherited Disease, McGraw-Hill, New York, 2001, pp. 3897–3933.

[5] S. Shinnar, H.S. Singer, Cobalamin C mutation (methylmalonic aciduria and homocystinuria) in adolescence. A treatable cause of dementia and myelopathy, N. Engl. J. Med. 311 (1984) 451–454.

[6] D.S. Rosenblatt, A.L. Aspler, M.I. Shevell, B.A. Pletcher, W.A. Fenton, M.R. Seashore, Clinical heterogeneity and prognosis in combined methylmalonic aciduria and homocystinuria (*cblC*), J. Inherit. Metab. Dis. 20 (1997) 528–538.

[7] G.A. Mitchell, D. Watkins, S.B. Melancon, D.S. Rosenblatt, G. Geoffroy, J. Orquin, M.B. Homsy, L. Dallaire, Clinical heterogeneity in cobalamin C variant of combined homocystinuria and methylmalonic aciduria, J. Pediatr. 108 (1986) 410–415.

[8] P. Augoustides-Savvopoulou, I. Mylonas, A.C. Sewell, D.S. Rosenblatt, Reversible dementia in an adolescent with *cblC* disease: clinical heterogeneity within the same family, J. Inherit. Metab. Dis. 22 (1999) 756–758.

[9] J.M. Powers, D.S. Rosenblatt, R.E. Schmidt, A.H. Cross, J.T. Black, A.B. Moser, H.W. Moser, D.J. Morgan, Neurological and neuropathologic heterogeneity in two brothers with cobalamin C deficiency, Ann. Neurol. 49 (2001) 396–400.

[10] O.A. Bodamer, D.S. Rosenblatt, S.H. Appel, A.L. Beaudet, Adult-onset combined methylmalonic aciduria and homocystinuria (*cblC*), Neurology 56 (2001) 1113.

[11] J.L. Van Hove, R. Van Damme-Lombaerts, S. Grunewald, H. Peters, D.B. Van, J.P. Fryns, J. Arnout, R. Wevers, E.R. Baumgartner, B. Fowler, Cobalamin disorder Cbl-C presenting with late-onset thrombotic microangiopathy, Am. J. Med. Genet. 111 (2002) 195–201.

[12] E. Roze, D. Gervais, S. Demeret, B.H. Ogier de, J. Zittoun, J.F. Benoist, G. Said, C. Pierrot-Deseilligny, F. Bolgert, Neuropsychiatric disturbances in presumed late-onset cobalamin C disease, Arch. Neurol. 60 (2003) 1457–1462.

[13] V. Guigonis, V. Fremeaux-Bacchi, S. Giraudier, R. Favier, D. Borderie, Z. Massy, B. Mougenot, D.S. Rosenblatt, G. Deschenes, Late-onset thrombocytic microangiopathy caused by *cblC* disease: association with a factor H mutation, Am. J. Kidney Dis. 45 (2005) 588–595.

[14] A.L. Boxer, J.H. Kramer, K. Johnston, J. Goldman, R. Finley, B.L. Miller, Executive dysfunction in hyperhomocysteinemia responds to homocysteine-lowering treatment, Neurology 64 (2005) 1431–1434.

[15] J.P. Lerner-Ellis, J.C. Tirone, P.D. Pawelek, C. Doré, J.L. Atkinson, D. Watkins, C.F. Morel, T.M. Fujiwara, E. Moras, A.R. Hosack, G.V. Dunbar, H. Antonicka, V. Forgetta, C.M. Dobson, D. Leclerc, R.A. Gravel, E.A. Shoubridge, J.W. Coulton, P. Lepage, J.M. Rommens, K. Morgan, D.S. Rosenblatt, Identification of the gene responsible for methylmalonic aciduria and homocystinuria, *cblC* type, Nat. Genet. 38 (2006) 93–100.

[16] M.J. Dillon, J.M. England, D. Gompertz, P.A. Goodey, D.B. Grant, H.A. Hussein, J.C. Linnell, D.M. Matthews, S.H. Mudd, G.H. Newns, J.W. Seakins, B.W. Uhlendorf, I.J. Wise, Mental retardation, megaloblastic anaemia, methylmalonic aciduria and abnormal homocysteine metabolism due to an error in vitamin B_{12} metabolism, Clin. Sci. Mol. Med. 47 (1974) 43–61.

[17] D.W. Bartholomew, M.L. Batshaw, R.H. Allen, C.R. Roe, D. Rosenblatt, D.L. Valle, C.A. Francomano, Therapeutic approaches to cobalamin-C methylmalonic acidemia and homocystinuria, J. Pediatr. 112 (1988) 32–39.

[18] Y. Brandstetter, E. Weinhouse, M.L. Splaingard, T.T. Tang, Cor pulmonale as a complication of methylmalonic acidemia and homocystinuria (Cbl-C type), Am. J. Med. Genet. 36 (1990) 167–171.

[19] P.B. Kazimiroff, D.M. Shaner, Methylmalonic acid and homocystinuria (cobalamin C mutant disease) presenting as acute paraparesis in an adolescent, Ann. Neurol. 30 (1991) 468.

[20] F.A. Wijburg, D.S. Rosenblatt, G.D. Vos, J.W. Oorthuys, L.G. van't Hek, B.J. Poorthuis, M.K. Sanders, R.B. Schutgens, Clinical and biochemical observations in a patient with combined Pompe disease and cblC mutation, Eur. J. Pediatr. 151 (1992) 127–131.
[21] M.T. Geraghty, E.J. Perlman, L.S. Martin, S.J. Hayflick, J.F. Casella, D.S. Rosenblatt, D. Valle, Cobalamin C defect associated with hemolytic-uremic syndrome, J. Pediatr. 120 (1992) 934–937.
[22] P. Russo, J. Doyon, E. Sonsino, H. Ogier, J.M. Saudubray, A congenital anomaly of vitamin B_{12} metabolism: a study of three cases, Hum. Pathol. 23 (1992) 504–512.
[23] R. Howard, I.J. Frieden, D. Crawford, T. McCalmont, M.L. Levy, D.S. Rosenblatt, L. Sweetman, S.I. Goodman, C. Ohnstad, K. Hart, M. Berrios, S. Packman, Methylmalonic acidemia, cobalamin C type, presenting with cutaneous manifestations, Arch. Dermatol. 133 (1997) 1563–1566.
[24] H.C. Andersson, E. Shapira, Biochemical and clinical response to hydroxocobalamin versus cyanocobalamin treatment in patients with methylmalonic acidemia and homocystinuria (*cblC*), J. Pediatr. 132 (1998) 121–124.
[25] C. Ellaway, J. Christodoulou, R. Kamath, K. Carpenter, B. Wilcken, The association of protein-losing enteropathy with cobalamin C defect, J. Inherit. Metab. Dis. 21 (1998) 17–22.
[26] B. Merinero, C. Perez-Cerda, M.J. Garcia, B. Chadefaux-Vekemans, P. Kamoun, C. Tonetti, J. Zittoun, C. Jakobs, M. Ugarte, Reliability of biochemical parameters used in prenatal diagnosis of combined methylmalonic aciduria and homocystinuria, Prenat. Diagn. 18 (1998) 947–952.
[27] G.M. Enns, A.J. Barkovich, D.S. Rosenblatt, D.R. Fredrick, K. Weisiger, C. Ohnstad, S. Packman, Progressive neurological deterioration and MRI changes in *cblC* methylmalonic acidemia treated with hydroxocobalamin, J. Inherit. Metab. Dis. 22 (1999) 599–607.
[28] H.C. Andersson, M. Marble, E. Shapira, Long-term outcome in treated combined methylmalonic acidemia and homocystinemia, Genet. Med. 1 (1999) 146–150.
[29] C.O. Harding, D.A. Pillers, R.D. Steiner, T. Bottiglieri, D.S. Rosenblatt, J. Debley, G.K. Michael, Potential for misdiagnosis due to lack of metabolic derangement in combined methylmalonic aciduria/hyperhomocysteinemia (*cblC*) in the neonate, J. Perinatol. 23 (2003) 384–386.
[30] P.J. Francis, D.M. Calver, P. Barnfield, C. Turner, R.N. Dalton, M.P. Champion, An infant with methylmalonic aciduria and homocystinuria (*cblC*) presenting with retinal haemorrhages and subdural haematoma mimicking non-accidental injury, Eur. J. Pediatr. 163 (2004) 420–421.
[31] R. Heidenreich, T. Grebe, M. Colville, K. Aleck, *cblC* defect in three Southwestern Native American infants, Mol. Genet. Metab. 84 (2005) 223.
[32] E.K. Tsina, D.L. Marsden, R.M. Hansen, A.B. Fulton, Maculopathy and retinal degeneration in cobalamin C methylmalonic aciduria and homocystinuria, Arch. Ophthalmol. 123 (2005) 1143–1146.
[33] D.S. Rosenblatt, B.A. Cooper, A. Pottier, H. Lue-Shing, N. Matiaszuk, K. Grauer, 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 (1984) 2149–2156.
[34] D. Watkins, N. Matiaszuk, D.S. Rosenblatt, Complementation studies in the cblA class of inborn error of cobalamin metabolism: evidence for interallelic complementation and for a new complementation class (cblH), J. Med. Genet. 37 (2000) 510–513.
[35] B. Ewing, L. Hillier, M.C. Wendl, P. Green, Base-calling of automated sequencer traces using phred. I. Accuracy assessment, Genome Res. 8 (1998) 175–185.
[36] D. Gordon, C. Abajian, P. Green, Consed: a graphical tool for sequence finishing, Genome Res. 8 (1998) 195–202.
[37] L.E. Maquat, Nonsense-mediated mRNA decay:splicing, translation and mRNP dynamics, Nat. Rev. Mol. Cell Biol. 5 (2004) 89–99.
[38] F.D. Ledley, H.L. Levy, S.L. Woo, Molecular analysis of the inheritance of phenylketonuria and mild hyperphenylalaninemia in families with both disorders, N. Engl. J. Med. 314 (1986) 1276–1280.
[39] P. Guldberg, H.L. Levy, R. Koch, C.M. Berlin Jr., B. Francois, K.F. Henriksen, F. Guttler, Mutation analysis in families with discordant phenotypes of phenylalanine hydroxylase deficiency. Inheritance and expression of the hyperphenylalaninemias, J. Inherit. Metab. Dis. 17 (1994) 645–651.
[40] K.M. Dipple, J.K. Phelan, E.R. McCabe, Consequences of complexity within biological networks: robustness and health, or vulnerability and disease, Mol. Genet. Metab. 74 (2001) 45–50.
[41] K.M. Dipple, E.R. McCabe, Modifier genes convert "simple" Mendelian disorders to complex traits, Mol. Genet. Metab. 71 (2000) 43–50.
[42] C.R. Scriver, P.J. Waters, Monogenic traits are not simple: lessons from phenylketonuria, Trends Genet. 15 (1999) 267–272.
[43] J. Vockley, P. Rinaldo, M.J. Bennett, D. Matern, G.D. Vladutiu, Synergistic heterozygosity: disease resulting from multiple partial defects in one or more metabolic pathways, Mol. Genet. Metab. 71 (2000) 10–18.