Atypical methylmalonic aciduria: frequency of mutations in the methylmalonyl-CoA epimerase (*MCEE*) gene.

by

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Abstract

Methylmalonic aciduria results from defects in the enzyme methylmalonyl-CoA mutase and from defects in the synthesis of the enzyme's cofactor adenosylcobalamin. Two patients who excrete methylmalonic acid have been shown to have a homozygous nonsense mutation in the methylmalonyl-CoA epimerase gene (*MCEE*). To further understand the causes of methylmalonic acid excretion, the *MCEE* gene was sequenced in 229 patients who excreted methylmalonic acid for which no cause was known. Mutations were detected in five patients. Fusion of fibroblast lines from two patients with a homozygous nonsense mutation in *MCEE* did not result in correction of [^{14}C]propionate incorporation toward control values while the defect in these fibroblasts was complemented by *mut*, *cblA*, and *cblB* fibroblasts. Transfection with wild-type *MCEE* cDNA resulted in correction of the biochemical phenotype in cells from both patients. These experiments support the hypothesis that a defective epimerase enzyme can be a cause of elevated methylmalonic acid excretion.

Résumé

L'acidurie méthylmalonique est reconnue comme étant le résultat d'un déficit de l'enzyme méthylmalonyl-CoA mutase (MCM) et de déficits dans la synthèse du cofacteur de MCM adénosylcobalamine. Deux patients excrétant de l'acide méthylmalonique avaient une mutation homozygote non-sens dans le gène codant pour méthylmalonyl-CoA épimérase (*MCEE*). Pour en savoir plus sur la cause de faibles excrétions d'acide méthylmalonique, le gène *MCEE* a été séquencé chez 229 patients qui excrétaient d'acide méthylmalonique sans cause connue. Des mutations dans *MCEE* ont été détectées chez cinq patients. La fusion des lignées de fibroblastes de deux patients homozygotes pour une mutation n'a pas corrigé l'incorporation de [^{14}C]propionate vers des valeurs normales tandis que le déficit de ces fibroblastes a été complémenté par des fibroblastes *mut*, *cblA*, et *cblB*. Une transfection avec l'ADNc de *MCEE* sauvage a corrigé le phénotype biochimique des cellules des deux patients. Ces expériences confirment l'hypothèse qu'un déficit de l'enzyme épimérase est une cause d'excrétion modérée d'acide méthylmalonique.

List of Abbreviations

AdoCbl	5'deoxyadenosylcobalamin
Cbl	cobalamin, vitamin B_{12}
CNCbl	cyanocobalamin
Cob(I)alamin	oxidation state of Co atom of cobalamin is +1
Cob(II)alamin	oxidation state of Co atom of cobalamin is +2
Cob(III)alamin	oxidation state of Co atom of cobalamin is +3
HC	haptocorrin
HPLC	high performance liquid chromatography
IF	intrinsic factor
MCM	methylmalonyl-CoA mutase
MeCbl	methylcobalamin
MS	methionine synthase
OHCbl	hydroxycobalamin
PBS	phosphate buffered saline
VOC	vicinal oxygen chelate superfamily

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CHAPTER 1

Introduction

1.1 Methylmalonic Aciduria

1.1.1 History of methylmalonic aciduria

Classic methylmalonic aciduria is marked by high levels of methylmalonic acid in both blood and urine and is associated with severe metabolic acidosis resulting in developmental delay, cognitive impairment, and even death in the early stages of life. Methylmalonic aciduria is one of the most frequent of the organic acidurias (Coulombe, et al., 1981).

The first patients with methylmalonic aciduria were described in the late 1960s (Oberholzer, et al., 1967; Rosenberg, et al., 1968; Stokke, et al., 1967). Oberholzer et al. described two cases of congenital metabolic acidosis in unrelated children, resulting from a block in the conversion of methylmalonic acid to succinic acid. The first patient was a male infant who presented with persistent mild acidosis and occasional acute episodes of severe metabolic acidosis. He suffered from intermittent vomiting associated with upper respiratory tract infection. He was admitted to hospital at eight months and was noted to have generalized hypotonia. There was persistent hepatomegaly, however the liver was shown to be functioning normally. The disorder was first thought to be an atypical form of renal tubular acidosis. He died at two years of age after an acute episode.

The second case reported was a female infant who presented from the first day of life with persistent acidosis (Oberholzer, et al., 1967). At presentation she appeared to be both physically and mentally retarded; additionally, hepatic enlargement was noted. Her clinical course appeared similar to that of the first case. She was found to have large

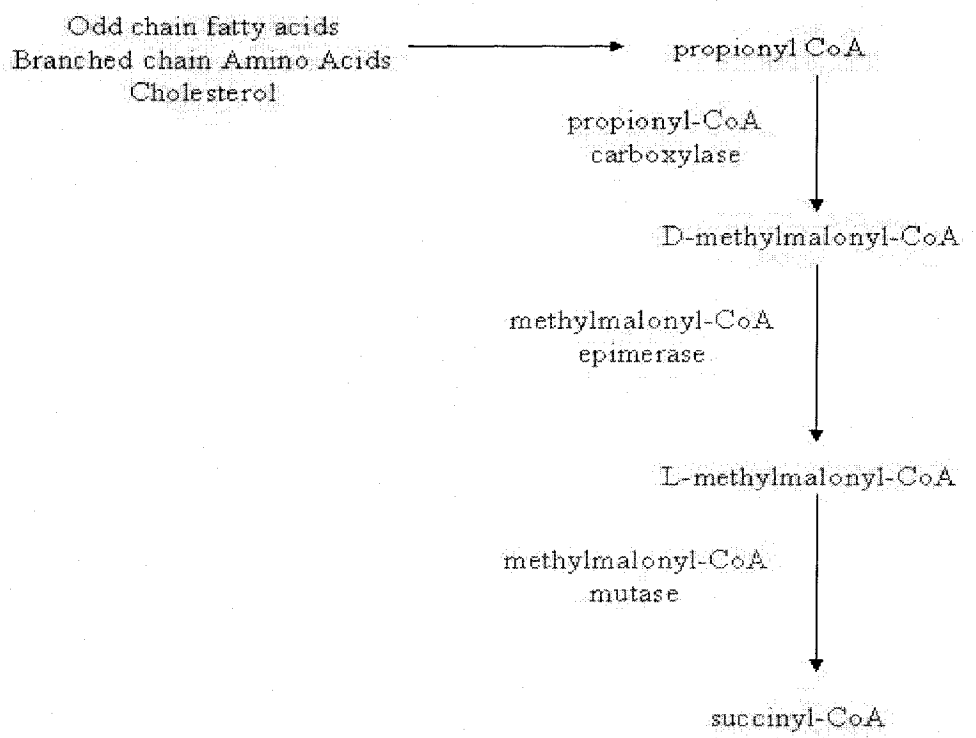
amounts of methylmalonic acid in her urine, blood, and cerebrospinal fluid. Ketosis was also noted, especially after protein loading. Normal serum vitamin B_{12} (cobalamin, Cbl) levels and extremely elevated methylmalonic acid excretion differentiated both these cases from those of vitamin B_{12} deficiency. The disease was proposed to be caused by an autosomal recessive inborn error of metabolism, likely by a defect in the enzyme methylmalonyl-CoA mutase (MCM).

Stokke et al. (1967) reported on a third patient with metabolic acidosis who produced enormous amounts of methylmalonic acid. This patient improved clinically when treated with a diet low in isoleucine, valine, threonine, and methionine, the four precursor amino acids to methylmalonyl-CoA. The tendency toward acidosis was reduced as well. Within the next year there were reports on a fourth patient with severe methylmalonic aciduria, intermittent hyperglycinemia, and long chain ketoacidosis (Rosenberg, et al., 1968). When this patient was given intramuscular injections of Cbl, methylmalonic acid excretion was reduced. It was concluded that a defect involving MCM was causal of the cases described and that the administration of large amounts of the MCM enzyme's cofactor 5'deoxyadenosylcobalamin (AdoCbl) might be beneficial in the long-term treatment of affected individuals.

In mammals propionyl-CoA is produced in the catabolism of certain essential amino acids, odd chain fatty acids, and cholesterol. Propionyl-CoA carboxylase (EC 6.4.1.3) carboxylates propionyl-CoA producing D-methylmalonyl-CoA. Methylmalonyl-CoA epimerase (EC 5.1.99.1) subsequently racemizes D-methylmalonyl-CoA to L-methylmalonyl-CoA, which is then converted to succinyl-CoA by MCM (EC 5.4.99.2)

using AdoCbl as a cofactor (Figure 1). It is now established that the biochemical defects responsible for methylmalonic aciduria are centered around MCM and its cofactor.

Figure 1
The propionate degradation pathway



Eight inborn errors of Cbl metabolism and utilization have been described through studies on patient fibroblasts (Gravel, et al., 1975; Mahoney, et al., 1975; Watkins and Rosenblatt, 1986; Watkins and Rosenblatt, 1988; Willard, et al., 1978). These inborn errors result in the presentation of isolated methylmalonic aciduria, isolated homocystinuria, or both combined. These groups have been designated *cblA* through *cblG* and *mut*. Patients with *cblA* through *cblG* are able to absorb Cbl normally and although their serum levels of the vitamin are within standard limits, they are unable to utilize it effectively. Methylmalonic aciduria is also associated with factors such as the lack of vitamin B_{12} in the diet, pernicious anemia, defects in the absorption of Cbl such as

hereditary intrinsic factor (IF) deficiency or Imerslund-Gräsbeck syndrome, and has also recently been associated with succinyl-CoA synthase deficiency (Carrozzo, et al., 2007; Ostergaard, et al., 2007).

1.1.2 Determination of cause of methylmalonic aciduria

Patients who excrete methylmalonic acid can be classified into different disease subgroups by studies using cultured fibroblasts. Diagnosis is achieved; 1) by measuring the ability of intact fibroblasts to incorporate label from [^{14}C]propionate into cellular macromolecules; 2) by measuring the synthesis of AdoCbl from exogenous [^{57}Co]cyanocobalamin (CNCbl); 3) by use of complementation assays to differentiate between disease groups. Patient cell lines will complement cell lines from all other inborn errors of Cbl metabolism; however they will not correct their own complementation class. There are a number of subjects who have elevated levels of methylmalonic acid in their blood and urine but who do not fit into any distinct category based on the above-mentioned criteria.

1.1.3 Treatments for patients with methylmalonic aciduria

Branched-chain amino acids are the primary source of methylmalonyl-CoA in humans. Therefore, patients may be placed on a low protein diet that minimizes the substrate for MCM. Patients with defects in the formation of the coenzyme AdoCbl can be treated with either CNCbl or hydroxycobalamin (OHCbl) in pharmacological doses (Coulombe, et al., 1981). Treatment is most successful when started before the appearance of clinical illness and can even be initiated prenatally (Ampola, et al., 1975).

Early diagnosis and treatment are important for the potential prevention of neurological damage or death.

1.1.4 Newborn screening for methylmalonic acid

The Massachusetts Metabolic Disorders Screening Program estimated the incidence of methylmalonic aciduria in Massachusetts to be at one in 48,000 births (Coulombe, et al., 1981). The Quebec Network of Genetic Medicine has included methylmalonic acid in their voluntary newborn screening program since 1975. The overall incidence of methylmalonic acid excretion in Quebec was found to be one in 43,600 children screened (Auray-Blais, et al., 1989) and symptomatic methylmalonic aciduria was found to occur in one of every 83,131 children screened (Sniderman, et al., 1999). In the Quebec screening program the lower cut-off for identifying cases of elevated methylmalonic acid excretion is 200 μmol/mmol creatinine during the newborn period.

The methods used for newborn screening have changed over the years. The Quebec Network of Genetic Medicine uses thin-layer chromatography on urine samples obtained at 21 days of age and positive cases are retested by gas chromatography on both urine and blood samples (Auray-Blais, et al., 1989). Symptomatic as well as benign cases are detected using these urine screening programs. The proper use of these screens is debated. Most severely symptomatic methylmalonic aciduria cases present clinically by one month of age, before test results are available, whereas benign excreters may never present clinically. It is not yet proven whether the overall outcome is better for those individuals identified through screening programs (Leonard, et al., 2003). More

recently, tandem mass spectrometry has been used as the method of screening for amino, organic, and fatty acid disorders (Zytkovicz, et al., 2001). This method is convenient because of its ability to pick up markers for multiple disorders using a single assessment, whereas methods such as chromatography detect a more limited number of diseases (Tarini, et al., 2006). With the advent of tandem mass spectrometry there is optimism regarding earlier detection of metabolic disorders such as methylmalonic aciduria. However, research is still underway in determining appropriate thresholds for identifying symptomatic versus asymptomatic cases and reasonable false positive rates. Expanded newborn screening has been associated with more positive outcomes such as decreased early mortality, less severe symptoms, and more favourable short-term neurodevelopment (Dionisi-Vici, et al., 2006). The positive effects of newborn screening are most beneficial for milder phenotypes, allowing for appropriate treatment in presymptomatic stages of disease.

1.1.5 Benign methylmalonic aciduria

Clinically benign (or asymptomatic) cases of methylmalonic aciduria have been detected through screening programs. A 1984 study estimated the frequency of benign methylmalonic aciduria at about one in 54,000 infants screened (Ledley, et al., 1984). Sniderman et al. (1999) reported the incidence of individuals with persistent benign methylmalonic aciduria in Quebec to be one in 49,900 births. The methylmalonic acid levels found in benign methylmalonic aciduria cases were much lower than those in symptomatic cases (Sniderman, et al., 1999).

The finding of individuals who are clinically asymptomatic but excrete moderate levels of methylmalonic acid has created problems in the interpretation of data obtained and in the counseling of patients. The majority of those found to have persistent mild excretion of methylmalonic acid have no obvious symptoms (Cederbaum and Vilain, 1999) thus creating uncertainty regarding what level of methylmalonic acid is considered within the normal range and of how to approach the treatment (or lack thereof) of those with benign methylmalonic aciduria. There are a number of reports on screening programs that discuss the varied clinical phenotype of patients who excrete methylmalonic acid at levels that are lower than in cases of classical methylmalonic aciduria but higher than in controls. There are also case reports of patients whose presentation does not fit within the classical definition of methylmalonic aciduria. A few of these reports are discussed below.

A metabolic screening program identified eight children who had persistent methylmalonic acid excretion but no apparent clinical abnormalities despite not being treated for their methylmalonic aciduria (Ledley, et al., 1984). These children showed no evidence of Cbl deficiency and lacked the secondary metabolites normally seen in symptomatic forms of methylmalonic aciduria. The methylmalonic acid levels in these eight subjects were higher than seen in normal newborn infants or even of those with dietary vitamin B_{12} deficiency, but were much lower than classical cases of methylmalonic aciduria. Complementation analysis was done for two of these cases rendering a diagnosis of *mut* deficiency (see section on isolated methylmalonic aciduria). As no complementation analysis was performed on the other six cases, there is no evidence whether or not they had the *mut* disorder. At the time of publication, the eight

children ranged in age from 18 months to 13 years and none had any unusual clinical presentations. A follow up study on six of these patients reported that they all remained symptom free at ages varying from eight to 20 years (Shapira, et al., 1991).

Information was assembled on infants who were found through the Quebec Network of Genetic Medicine screen to excrete methylmalonic acid. They reported on 126 cases of methylmalonic acid excretion in which methylmalonic acid levels were less than 1400 μmol/mmol creatinine, a level that is higher than the proposed control cut-off of 200 μmol/mmol creatinine. Sixty-five of the 126 cases were considered to be transient, meaning that the methylmalonic acid excretion resolved by one year of age or responded to vitamin B_{12} therapy. A few cases had symptomatic methylmalonic aciduria despite the lower methylmalonic acid levels (<1400 μmol/mmol creatinine); most of these cases were diagnosed with known inborn errors of B_{12} metabolism. Another group had moderate persistent methylmalonic acid excretion (<1400 μmol/mmol creatinine) that was asymptomatic. It was hypothesized that liver immaturity may contribute to the etiology of this abnormality. Complementation studies were done on some of these asymptomatic cases and one patient was given a *mut*$^-$ diagnosis, while the cause of the methylmalonic acid excretion in others remains unknown. The authors suggested that the asymptomatic group may have milder deficiencies of MCM or Cbl synthesis that cannot be detected by available assays, or that a mutation of another unknown locus is involved (Sniderman, et al., 1999).

A four-year-old girl was reported to have a severe clinical phenotype despite having the biochemical features of mild methylmalonic acidemia (Shapira, et al., 1991). The child had an unremarkable developmental course until age four when she was

admitted to hospital with a four-day fever, vomiting, abdominal pain, and lethargy. There was a semicomatose episode characterized by ketoacidosis and hyperammonemia. Her urinary methylmalonic acid levels were relatively low, and consistent with levels seen in asymptomatic methylmalonic aciduria, although values were higher during episodes of vomiting. She had a second episode two years later characterized by ketonuria and hyperammonemia but no metabolic acidosis. This patient was shown through complementation analysis to have the *mut*$^-$ defect. The authors proposed that benign methylmalonic acid excretion might be a mild phenotype, associated with *mut* deficiency. This case indicates that mild methylmalonic acid excretion is not necessarily a benign disorder and is at least one instance where the correlation between methylmalonic acid levels and severity of symptoms is not directly proportional.

Treacy et al. (1993) reported on a male patient who was found through a newborn screening program to have elevated levels of methylmalonic acid in his urine (294 μmol/mmol creatinine), however at the time of the report he was free of symptoms. This patient was given the diagnosis of *mut*$^-$ through complementation analysis and was treated from early infancy with a low protein diet. At follow up at age five the patient had massive methylmalonic acid levels (urine 1558 μmol/mmol, control 5 μmol/mmol; and serum methylmalonic acid mean 294 μmol/L, control <17 μmol/L) indicating severe methylmalonic aciduria, however the child still had no signs of metabolic decompensation despite five years of exposure to these metabolites. At that time the case was thought to further complicate the topic of asymptomatic methylmalonic aciduria, suggesting that methylmalonic acid and propionate derivatives cannot fully explain the metabolic and neurotoxic effects typically seen in methylmalonic aciduria patients.

Interestingly, this patient presented again at age seven in severe metabolic crisis (Treacy, et al., 1996). The second report on this patient demonstrates that lack of clinical disease at the time methylmalonic acid is identified is not an indication that the individual will not become symptomatic at a later time. At the time of the second report this patient was noted to have glutathione deficiency. In addition, this patient was found to have mutations in the *MUT* gene (homozygous for c.2150C>T, G717V) (Worgan, et al., 2006).

Overall there are variable clinical outcomes in individuals found through metabolic screening programs to excrete moderate levels of methylmalonic acid. Some benign cases have been diagnosed with *mut*$^{-}$ deficiency. Many benign methylmalonic aciduria cases with and without diagnoses remain asymptomatic at follow up. The range in outcome from asymptomatic to severely affected creates uncertainties in the treatment and counselling of patients and highlights the need for further investigations into the nature of this disorder.

1.2 Differential Diagnosis of Methylmalonic Aciduria

1.2.1 Methylmalonic aciduria due to dietary vitamin B_{12} deficiency

1.2.1-1 Cobalamin (vitamin B_{12})

Cbl is an organometallic compound that is required for cellular intermediary metabolism. The vitamin is composed of a central cobalt atom surrounded by a planar corrin ring. A complex side chain consisting of a 5, 6-dimethylbenzimidazole base extends down from the corrin plane. The cobalt atom is linked to one of the nitrogens of the benzimidazole group by coordination in the bottom axial position and also to 4 nitrogenes of the corrin ring (Hodgkin, et al., 1956).

Several different substituents can be coordinated to the upper axial position in order to complete the molecule. The most common commercial form of the vitamin, CNCbl, is formed by the complexing of a cyanide ion to the cobalt atom (Rosenblatt and Fenton, 2001). This form of the vitamin is an artefact of isolation and does not occur naturally in microorganisms or animal tissues. There are four common forms of Cbl routinely isolated from mammalian tissue: OHCbl, methylcobalamin (MeCbl), AdoCbl, (Rosenblatt and Fenton, 2001) and glutathionylcobalamin (Jacobsen and Green, 1986). As well, complexes of other sulfhydryls with Cbl have been documented. OHCbl is thought by some to be the physiological form of the vitamin that is taken up by the cell. MeCbl and AdoCbl are the only compounds in nature known to have a direct covalent carbon-cobalt bond. In mammalian systems, AdoCbl and MeCbl are the required cofactors for the enzymes MCM and methionine synthase (MS) respectively.

1.2.1-2 Cobalamin requirements and distributions

The mean value for the total body content of vitamin B_{12} in human adults ranges between 2 to 5 mg, 1.5 mg of which is contained in the liver (Linnell, 1975). The distribution of Cbl in the organs is varied with the concentrations highest in the liver and kidneys, organs known to have high levels of the Cbl-dependent enzymes. The normal range of serum Cbl is 200-900 pg/ml (147-664 pmol/L) with the majority (425-450 pg/ml or 314-332 pmol/L) transported by haptocorrin (HC, formerly called transcobalamin I) (Allen, 1992). Studies have shown the recommended daily intake of Cbl to be 0.1 to 0.25 μg per day (Herbert, 1988).

Mammals require Cbl for normal metabolism due to the two mammalian enzymes, MCM and MS, which use it as a cofactor. Humans are unable to synthesize Cbl; therefore it must be obtained from animal products in which it is widespread and derived from bacteria (Herbert, 1988). Cbl is available in meat (especially liver), egg yolk, and even dairy products provide a moderate source. Cbl is widely distributed in animal tissues where it is found in varied concentrations (Beck, 1982). However, Cbl is synthesized only by microorganisms, found in the rumen and small intestine of animals, or by microorganisms found in soil and water. Interestingly human colon bacteria produce a large amount of Cbl but it is not absorbed through the colon and therefore the vitamin must be obtained from exogenous sources of animal products. Plants do not synthesize Cbl, however due to contamination by bacteria, traces are sometimes found in plant foods.

1.2.1-3 Dietary cobalamin deficiency

A lack of Cbl in the diet can cause methylmalonic aciduria and clinical symptoms such as megaloblastic anemia and neurological degeneration. The biological half-life of Cbl is between 460 to 230 days (Gräsbeck, 1959). Cbl dietary deficiency may not result in clinical symptoms for years; up to 90 percent of the 1-10 μg of biliary Cbl secreted daily is reabsorbed. The high degree of reabsorption explains why individuals who stop consuming vitamin B_{12} may not present clinically for up to 20 years, but why those who are no longer able to absorb the vitamin show symptoms in less than three years (Herbert, 1988). In Western communities dietary Cbl deficiency is seen almost exclusively in vegans.

1.2.2 Methylmalonic aciduria due to defective cobalamin absorption

1.2.2-1 Cobalamin absorption

The absorption and transport of Cbl in mammals is a complex process that requires gastric, ileal, and pancreatic components. Absorption of vitamin B_{12} begins in the acidic environment of the stomach when it is released from dietary protein. Once released, Cbl binds to proteins of salivary and gastric origin known as haptocorrins, or R-binders (Allen, et al., 1978). Pancreatic proteases digest the R-binders releasing Cbl into the upper duodenum of the small intestine where it complexes with IF, a glycoprotein synthesized by the gastric parietal cells (Levine, et al., 1980). In the presence of calcium ions, the IF-Cbl complex then interacts with cubam, an ileal IF-Cbl receptor complex on ileal mucosal cells. Cubam is composed of two proteins: cubulin, a large membrane protein of 460 kDa, and amnionless, a transmembrane protein of approximately 50 kDa

(Fyfe, et al., 2004). Amnionless binds the amino terminal third of cubulin and directs subcellular localization and endocytosis of the IF-Cbl ligand into the enterocyte where the complex then dissociates (Fyfe, et al., 2004).

Cbl is transported across the enterocyte basal membrane into the portal blood bound to transcobalamin which acts as the transport protein for the absorbed vitamin (Katz and O'Brien, 1979). The circulating transcobalamin/Cbl complex is recognized by a specific high-affinity plasma membrane receptor and brought into the lysosomes via adsorptive endocytosis (Youngdahl-Turner, et al., 1978). A lysosomal transport system allows for the release of Cbl into the cytoplasm (Idriss and Jonas, 1991). Cbl will then either remain in the cytosol to be converted to MeCbl and act as a cofactor for the MS enzyme or enter the mitochondrion where it will be adenosylated to AdoCbl, the cofactor for MCM.

1.2.2-2 Pernicious anemia

Pernicious anemia is an autoimmune disease that results in the destruction of stomach parietal cells that produce IF and is a major cause of Cbl deficiency in non-vegan adults in Western communities. Patients with this disorder have autoantibodies to IF and to parietal cells (Toh, et al., 1997) resulting in the inability to absorb Cbl from the diet. Pernicious anemia is controlled by treatment with Cbl.

1.2.2-3 Hereditary Intrinsic Factor deficiency

Hereditary IF deficiency is known to result from defective or absent IF, causing impaired Cbl absorption. Those affected present between one and five years of age with developmental delay and megaloblastic anemia (Cooper and Rosenblatt, 1987; Yang, et al., 1985). Patients with IF deficiency have been found to have mutations in the gastric

IF gene (*GIF*) (Tanner, et al., 2005), a defect that is associated with the failure to produce or secrete any immunologically recognizable IF (Spurling, et al., 1964), or the production of immunologically recognizable IF that is biologically inert (Katz, et al., 1972). In these patients serum Cbl levels are very low, and serum autoantibodies to IF are absent, and gastric function is normal unlike in pernicious anemia (Rosenblatt and Fenton, 2001). When exogenous Cbl is mixed with normal human gastric juice as a source of IF, absorption corrects and proceeds normally (Rosenblatt and Fenton, 2001). Clinical symptoms are similar to those of dietary vitamin B_{12} deficiency and pernicious anemia.

1.2.2-4 Imerslund-Gräsbeck syndrome

Imerslund and Gräsbeck independently described a defect affecting the ileal phase of absorption in which IF functions correctly but vitamin B_{12} absorption is abnormal (Gräsbeck, et al., 1960; Imerslund, 1960). In this disorder, known as the Imerslund-Gräsbeck syndrome, it is the intestinal Cbl absorption into the enterocyte that is defective. Imerslund-Gräsbeck syndrome patients have been shown to harbour mutations in either the genes coding for cubulin (*CUBN*) or amnionless (*AMN*) (Aminoff, et al., 1999; Tanner, et al., 2003). The disorder is characterized by clinical symptoms similar to those of hereditary IF deficiency but is differentiated by having a normal IF function. Another distinction is that many Imerslund-Gräsbeck patients develop proteinuria (Mackenzie, et al., 1972).

1.2.2-5 Transcobalamin deficiency

Transcobalamin deficiency is a rare autosomal recessive disorder in which transcobalamin is defective or absent. Most patients with transcobalamin deficiency have no immunologically detectable transcobalamin in the plasma. However, serum levels of

Cbl may be normal because the majority of serum Cbl is transported by HC. Those affected usually present within the first two months of life with failure to thrive, megaloblastic anemia and eventually immunologic deficiency (Cooper and Rosenblatt, 1987). Neurologic manifestations have been documented and may develop in the absence of adequate Cbl and folate treatment (Hall, 1992). Patients with transcobalamin deficiency have been found to have mutations in the human *TCN2* gene (Li, et al., 1994).

1.2.3 Causes of isolated methylmalonic aciduria

Inherited defects that affect the enzymatic conversion of L-methylmalonyl-CoA to succinyl-CoA are also known to result in the accumulation of methylmalonic acid in the blood and urine. Isolated methylmalonic aciduria is caused by a defect in MCM (*mut*) or in the synthesis of the enzyme's cofactor (*cblA, cblB,* and *cblD* variant-2/*cblH*). Cell cultures from patients with isolated methylmalonic aciduria show a reduced ability to incorporate labelled propionate, a precursor of methylmalonyl-CoA, into cellular macromolecules. The incorporation of propionate is a measure of the function of the pathway for conversion of propionyl-CoA to succinyl-CoA.

The most common clinical symptoms in patients with isolated methylmalonic aciduria are lethargy, failure to thrive, recurrent vomiting, dehydration, respiratory distress, and muscular hypotonia (Matsui, et al., 1983). An accumulation of large amounts of methylmalonic acid in the blood is present even in the absence of clinical acidosis. As well, elevated levels of ammonia, ketones, glycine, and glucose may be present in the blood.

1.2.3-1 Methylmalonyl-CoA mutase deficiency

Patients with the *mut* disorder develop isolated methylmalonic aciduria that is not responsive to pharmacologic doses of Cbl. *In vitro* synthesis of AdoCbl from labelled CNCbl is usually in the normal range in *mut* fibroblasts. Patients present very early in life and usually within four weeks of birth (Matsui, et al., 1983). Patients diagnosed with *mut* deficiency through complementation analysis have been shown to have mutations in the *MUT* gene coding for the MCM enzyme.

Within the *mut* complementation class there are two subgroups designated mut^0 and mut^- (Morrow, et al., 1978; Willard and Rosenberg, 1977). These subgroups can be differentiated by a number of measures and investigators may define these groups based on different criteria. For example, some may differentiate the two groups based on enzymatic response to AdoCbl: MCM activity in mut^- lines, measured by propionate incorporation or by mutase enzyme assay, can be induced by increasing AdoCbl concentration in experimental cultures. However, mut^0 lines do not display increased MCM activity under these conditions. Others may differentiate the groups based on the response to human MCM antibody, with mut^- cell extracts showing more cross reaction to antibody of human MCM in comparison to mut^0 cells (Kolhouse, et al., 1981). Overall mut^0 and mut^- can be differentiated by complete versus partial absence of functional apoenzyme.

A further distinction is that mut^0 patients usually have earlier onset disease and a poorer prognosis than mut^- patients (Shevell, et al., 1993). A panel of 20 mutase patients were studied, 11 of which were mut^0 and 9 of whom were mut^-. All mut^0 patients had early presentations of ketoacidosis within the neonatal period. This invariable neonatal

presentation in *mut*^{0} patients was in accordance to that reported by Matsui et al. in 1983. Overall *mut*$^-$ patients became symptomatic in infancy or early childhood (between two months and four years) and had milder deficits. It is important to note that although there are broad correlations between the mutase classes and phenotypes, good outcomes are possible among *mut*^{0} as are poor outcomes in *mut*$^-$. As well, early diagnosis and proper treatment have led to better prognosis than in the past.

The *mut* complementation group accounts for the largest cohort of patients with isolated methylmalonic aciduria. At least 178 mutations in the *MUT* gene on chromosome 6p21 have been identified in *mut* patients (Lempp, et al., 2007; Worgan, et al., 2006). A study of 160 *mut* patients revealed 116 different mutations (Worgan, et al., 2006). Certain regions of the *MUT* gene are thought to be hotspots for mutations as a number of mutations occur in the same or adjacent nucleotides. As well, most mutations were located in one of the two functional domains of the MCM enzyme. Certain mutations have been seen primarily in individuals of the same ethnic background: p.R108C in Hispanic patients, p.G717V in Black patients, and p.G544X and p.G427D in Asian patients. Other mutations such as p.R228X, p.R369H, p.R152X and p.R727X were seen in patients of varied ethnic background (Worgan, et al., 2006).

1.2.3-2 *CblA, cblB,* and *cblD* variant-2 deficiencies

Patients with the c*blA* and *cblB* disorders develop isolated methylmalonic aciduria that can be responsive to pharmacological doses of Cbl (Rosenblatt and Fenton, 2001). Clinically, c*blA* and c*blB* patients usually present within the first year of life with similar symptoms to *mut* patients, for example recurrent vomiting, failure to thrive and hypotonia (Matsui, et al., 1983).

In fibroblasts from these patients, synthesis of AdoCbl from labelled CNCbl is deficient in the presence of normal MeCbl synthesis (Mahoney, et al., 1971). In the presence of exogenous reducing agent the synthesis of AdoCbl from labelled CNCbl is normal in both *cblA* and *cblD* variant- 2/*cblH* extracts however not in those from *cblB* (Mahoney, et al., 1975). As well, the functional activity of MCM is decreased in extracts from these patients; however the specific activity of the enzyme is normal.

The *cblA* disorder is caused by a defect in the MMAA protein. The gene coding for this protein (*MMAA*) was identified through the analysis of prokaryotic gene arrangements (Dobson, et al., 2002b). The function of the *MMAA* gene product is currently not certain. However, *MeaB* an MMAA homologue in *Methylobacterium extorquens*, acts as a factor protecting MCM from irreversible inactivation (Korotkova and Lidstrom, 2004). It has been recently shown that *MeaB* interacts with MCM directly (Padovani and Banerjee, 2006) and other work has suggested that Cbl becomes associated with MCM even when MMAB is inactive (Moras, et al., 2007).

The *cblB* disorder corresponds to a defect in the *MMAB* gene that codes for the ATP:adenosyltransferase which catalyzes the last step in AdoCbl synthesis (Fenton and Rosenberg, 1981) (see section on AdoCbl). The *MMAB* gene was also identified on the basis of gene arrangement in bacteria (Dobson, et al., 2002a). The crystal structure in *Thermoplasma acidophilim* was determined and the enzyme was shown to be a trimer, with each subunit composed of a five-helix bundle (Saridakis, et al., 2004).

The formerly known *cblH* complementation group consisting of one patient with isolated methylmalonic aciduria was described in 2000 (Watkins, et al., 2000). Complementation experiments with fibroblasts from patients with isolated methylmalonic

aciduria distinguished this patient as a distinct complementation group. This patient was biochemically and clinically similar to *cblA* patients but no mutations in *MMAA* were found through sequencing analysis. Recent studies have increased our understanding of the *cblD* defect (see below) and complementation studies have shown that the sole *cblH* patient in fact belongs to the *cblD* variant-2 subgroup (personal communication, Dr. David Watkins and Dr. Brian Fowler).

The *cblD* disorder was initially described in a pair of siblings with combined methylmalonic aciduria and homocystinuria (Willard, et al., 1978). However, it was recently shown through complementation analysis that two patients with isolated homocystinuria and one patient with isolated methylmalonic aciduria did not complement the original *cblD* lines, identifying them as part of this complementation group. These patient groups were named *cblD* variant-1 and *cblD* variant-2 respectively (Suormala, et al., 2004). The *cblD* disorder has been reported in only six patients and the clinical course appears quite varied. The gene corresponding to the *cblD* defect has recently been identified (personal communication, Dr Brian Fowler).

1.2.3-3 Succinyl-CoA synthase deficiency

Mutations in the *SUCLA2* gene leading to succinyl-CoA synthase deficiency were described in a pedigree of four patients who had a Leigh-like encephalomyopathy, deafness, and mitochondrial DNA depletion (Elpeleg, et al., 2005). Besides the presence of lactic acidosis, urine organic acid was not reported. This discovery prompted the testing of patients with undiagnosed methylmalonic aciduria for mutations at the *SUCLA2* locus, since the reaction catalyzed by succinyl-CoA synthase in the tricarboxylic acid cycle represents a distal step of the methylmalonic acid pathway

(Carrozzo, et al., 2007; Ostergaard, et al., 2007). These studies identified mutations in the *SUCLA2* gene of patients who excreted methylmalonic acid.

1.2.3-4 Methylmalonyl-CoA epimerase deficiency

Historically, it was hypothesized that a defect in the methylmalonyl-CoA epimerase enzyme could cause isolated methylmalonic aciduria, however there was much debate over this topic. Recently, two cases of methylmalonic aciduria were attributed to a defective epimerase enzyme; a homozygous nonsense mutation in the *MCEE* gene was identified in two probands (Bikker, et al., 2006; Dobson, et al., 2006). These are the first confirmed cases of epimerase deficiency in humans; the subject of an earlier report thought to have a defect in the epimerase (Kang, et al., 1972) was later shown to have MCM deficiency (Rosenberg and Scriver, 1980).

1.3 Methylmalonyl-CoA Mutase

1.3.1 Methylmalonyl-CoA mutase

MCM is one of the two Cbl dependant enzymes that are found in both mammals and bacteria (Mellman, et al., 1977). This enzyme catalyzes the conversion of methylmalonyl-CoA to succinyl-CoA, an important step in the propionate degradation pathway. This pathway may also function in reverse in certain microbial species: In the bacterion *Propionibacterium shermanii,* MCM has been shown to be involved in the fermentation of propionate to succinate (Allen, et al., 1964).

1.3.2 Methylmalonyl-CoA mutase structure

The crystal structure of MCM from *Propionibacterium shermanii* is described as an αβ heterodimer of 150 kDa (80 kDa and 728 residues in the α-chain and 70 kDa and 638 residues in the β-chain) containing one binding site for acyl-CoA and B_{12} per dimer (Mancia, et al., 1996). The active site is present in the α-subunit and only this larger subunit is capable of binding the AdoCbl cofactor and the CoA ester substrates (Thoma and Leadlay, 1998). The substrate-binding site was identified within the TIM-barrel $(\beta/\alpha)_8$ domain (Mancia, et al., 1996). The AdoCbl coenzyme is bound in a domain that shares a similar fold to the MeCbl binding domain of MS. The mature human MCM enzyme and the α-subunit of *Propionibacterium shermanii* MCM have approximately 65 percent similarity. Using the crystal structure of the *Propionibacterium shermanii* MCM enzyme Thoma and Leadley (1996) were able to derive the 3-D structure of human MCM.

Human MCM forms an α_2 homodimer, each unit being 718 amino acids long (78.5 kDa) and each mole of subunit binding one mole of AdoCbl. The product of the nuclear gene *MUT* is transported into the mitochondrial matrix where its 32 amino acid mitochondrial leader sequence is cleaved. There are two major domains of the enzyme; the N-terminal $(\beta\alpha)_8$ TIM barrel (residues 88-422) containing the substrate binding domain, and the C-terminus (residues 578-750) of the protein containing the Cbl-binding domain. The N-terminal extended segment (residues 32-87) is involved in subunit-subunit interaction. The substrate and AdoCbl binding domains are connected by a long linker region (residues 423-577) (Jansen, et al., 1989; Thoma and Leadlay, 1996; Worgan, et al., 2006).

The molecular cloning of L-methylmalonyl-CoA mutase (*MUT*) was performed by screening human placenta and liver cDNA expression libraries for MCM clones using antibody to human placenta MCM (Ledley, et al., 1988a). The authenticity of the clone was confirmed by gene transfer of the recombinant clone into a cultured cell line with low MCM activity, with the transfected cells expressing up to five-fold increased MCM enzymatic activity compared to untransfected controls. In further studies the MCM gene was localized to chromosome 6p12-21.2 by the mapping of somatic-cell hybrid cell lines and *in situ*-hybridization (Ledley, et al., 1988b). The *MUT* gene consists of 13 exons spanning over 35 Kb of genomic DNA (Nham, et al., 1990). The first exon is noncoding and no intron separates the mitochondrial targeting sequences and the mature apoenzyme (Nham et al., 1990).

1.3.3 Adenosylcobalamin (AdoCbl)

AdoCbl, the cofactor of the mitochondrial enzyme MCM, is a Cbl derivative in which a 5'-deoxyadenosyl group is linked covalently to the central cobalt atom. In mammals the role of AdoCbl is in the generation of a free radical that initiates catalysis of the reaction in which methylmalonyl-CoA is converted to succinyl-CoA (Marsh, 1995).

The intracellular synthesis of AdoCbl has been described in *Clostridium tetanomorphum* bacteria (Vitols, et al., 1966; Walker, et al., 1969), and in HeLa cells (Kerwar, et al., 1971). However, certain aspects of AdoCbl synthesis remain poorly understood. In *Clostridium tetanomorphum* bacteria, AdoCbl is derived from cob(III)alamin by the action of three enzymes; two reductases and an adenosyltransferase. Both reductases are flavoproteins that require NADH as a cofactor. The first, NADH:cob(III)alamin oxidoreductase (EC 1.16.1.3), converts cob(III)alamin to cob(II)alamin. The second, NADH:cob(II)alamin oxidoreductase (EC 1.6.1.4), catalyzes the further reduction to cob(I)alamin. The synthesis of cofactor AdoCbl is completed when the cob(I)alamin adenosyltransferase (ATP:cob(I)alamin adenosyltransferase, EC 2.5.1.17) acts on its substrates cob(I)alamin and ATP.

Studies have suggested that Cbl metabolism is similar in the mammalian cell and that both AdoCbl synthesis and activity take place in the mitochondria (Fenton and Rosenberg, 1981; Mahoney, et al., 1975; Mahoney and Rosenberg, 1971). However, it is unknown where the reduction steps are localized and in which oxidation state Cbl enters the mitochondria. The mechanism of Cbl entry into the mitochondria has also yet to be determined.

1.4 Methylmalonyl-CoA Epimerase

1.4.1 Methylmalonyl-CoA epimerase

The methylmalonyl-CoA epimerase (racemase) enzyme is involved in the catabolism of propionyl-CoA. The methylmalonyl-CoA epimerase requirement in the conversion of propionyl-CoA to succinyl-CoA was first described by Mazumder et al (1961). Methylmalonyl-CoA epimerase activity has been identified in a wide range of species (Leadlay, 1981; Stabler, et al., 1985). A multiple sequence alignment with available prokaryotic and eukaryotic epimerases revealed that there is a 65 percent amino acid sequence similarity between epimerases from humans, mice, and *Methylobacterium extorquens* (Kuhnl, et al., 2005). More than one sixth of the residues are conserved across a wide range of species. In prokaryotes, epimerases are involved in the propionate fermentation and in autotrophic CO_2 fixation via the 3-hydroxypropionate pathway (Herter, et al., 2002; Herter, et al., 2001). Methylmalonyl-CoA epimerase is also part of the glyoxylate regeneration pathway in the bacterium *Methylobacterium extorquens* (Korotkova, et al., 2002). As well, epimerases are involved in the biosynthesis of polyketide antibiotics that requires propionate and methylmalonate units. These antibiotics have broad therapeutic applications (Pfeifer and Khosla, 2001).

The epimerase gene, the first Cbl related gene to be identified on the basis of prokaryotic gene arrangements, was localized to chromosome 2p13.3 (Bobik and Rasche, 2001). The human *MCEE* gene consists of three exons and codes for 176 amino acids. Based on the MitoProt software program there is a 95 percent probability of import into the mitochondria where a 29 amino acid leader sequence is cleaved.

Methylmalonyl-CoA epimerase has been suggested to be a part of the vicinal oxygen chelate (VOC) superfamily (Armstrong, 2000; Babbitt and Gerlt, 1997; Bernat, et al., 1999). This family has similarities in the catalytic mechanism of its enzyme members. The mechanism includes proton abstraction and transfer as well as substrate binding to a metal ion in order to stabilize the transition state (Armstrong, 2000; McCarthy, et al., 2001). The McCarthy studies (2001) also confirmed the enzyme's membership to the VOC enzyme superfamily by revealing its structural and functional similarity with glyoxalase I (a VOC superfamily member). The modes in which their monomers assemble link them both to an evolutionary pathway of the superfamily and suggest that the evolution of these proteins probably involved gene duplication, gene fusion, and domain swapping from a common precursor (McCarthy, et al., 2001).

1.4.2 The methylmalonyl-CoA epimerase reaction

Mazumder et al (1961) suggested that the racemization reaction occurs either by a shift in the α-hydrogen atom or by transfer of the CoA moiety from one carboxyl group to the other. It was proven that the former reaction in fact occurs, involving a rearrangement of a hydrogen atom about carbon atom 2 (C2). This α-hydrogen atom is lost and subsequently a proton is incorporated from the medium (Mazumder, et al., 1962; Overath, et al., 1962).

Studies on *Propionibacterium shermanii* epimerase in tritiated water revealed that two bases are involved in epimerization reaction (Fuller and Leadlay, 1983). In this reaction, one base abstracts a proton from C2 from one side of the methylmalonyl-CoA substrate and the C2 configuration inverts. The conjugate acid of the second base then

protonates the C2 from the opposite side. Stabilization of the anion intermediate is achieved by the substrate binding a metal in the active site. Methylmalonyl-CoA epimerase is activated by incubation with divalent ions, for example by Co^{2+}or Mn^{2+} in *Propionibacterium shermanii,* and may also be inactivated by treatment with metal-chelating agents (Leadlay, 1981).

Bobik and Rasche (2003) developed a high performance liquid chromatography (HPLC) assay to study the methylmalonyl-CoA epimerase reaction. In this assay, methylmalonyl-CoA epimerase activity is measured by monitoring the disappearance of methylmalonyl-CoA by HPLC. The substrate used in this assay was a commercially available (2R, S)-methylmalonyl-CoA and required the depletion of the 2R epimer from the mixture before the addition of methylmalonyl-CoA epimerase. This depletion was accomplished by the addition of holo-MCM to the reaction mixture, eliminating the 2R isomer that is MCM specific. The activity of methylmalonyl-CoA epimerase is then measured using HPLC to monitor the consumption of 2S methylmalonyl-CoA.

1.4.3 Methylmalonyl-CoA epimerase crystal structure

The crystal structures of methylmalonyl-CoA epimerase for *Propionibacterium shermanii* and *Caenorhabditis elegans* have been described (Kuhnl, et al., 2005; McCarthy, et al., 2001). The enzyme was shown to form a dimer; each monomer consisting of two βαβββ modules that pack edge to edge creating an 8-stranded β sheet. The β sheet curves to create the specific metal binding active site (McCarthy, et al., 2001). The crystal structure confirmed the mode of substrate binding and revealed the binding site for a divalent metal ion, for example Co^{2+}. In *Propionibacterium shermanii*

the metal ion is bound deep within the active site cleft, coordinated to the side chains of amino acids His12, Gln65, His91, and Glu141. Modeling of 2-methylmalonate into the active site of the enzyme identified two glutamate residues as the likely essential bases for the epimerase reaction; Glu48 to abstract the proton and Glu141 to donate the proton. It was proven through sequence alignment that the Glu141 is conserved in all known methylmalonyl-CoA epimerases, whereas Glu48 is replaced by a valine or threonine (Kuhnl, et al., 2005).

1.4.4 Methylmalonyl-CoA epimerase expression and distribution

Kuhnl et al (2005) studied the expression and distribution of the epimerase gene in *Caenorhabditis elegans* (*mce-1*). *Mce-1* is expressed prominently in body wall muscles and moderately in parts of the pharynx and hypodermis. The subcellular distribution of *mce-1* shows that it is not distributed evenly; it has a distinct dotted appearance consistent with mitochondrial localization.

1.4.5 Methylmalonyl-CoA epimerase debate

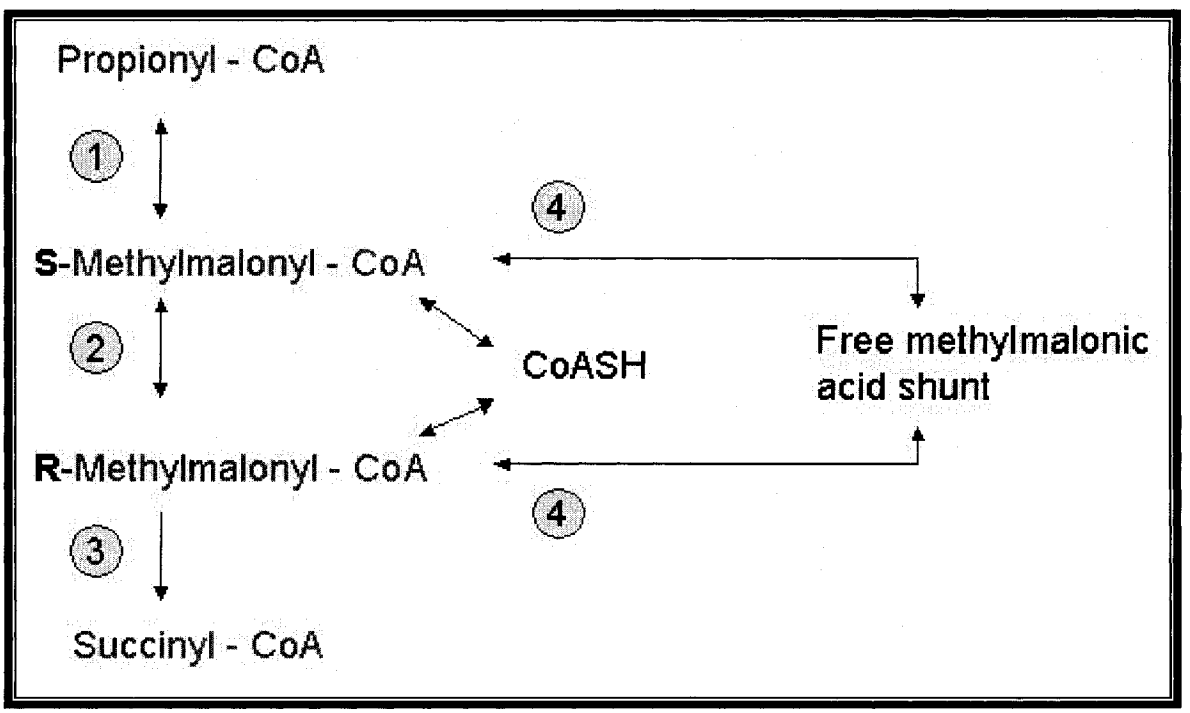
There is an ongoing debate regarding the necessity of the methylmalonyl-CoA epimerase enzyme, as studies have suggested that an alternate non-enzymatic route for the conversion of the D to the L isomer exists in parallel to epimerase. These studies suggest that conversion from D to L-methylmalonyl-CoA can occur spontaneously as well as by catalysis involving the epimerase enzyme. It has been argued that spontaneous conversion might be sufficient to maintain propionate catabolism in the absence of

functional epimerase. Other studies have highlighted the importance of methylmalonyl-CoA epimerase in normal metabolism or under conditions of metabolic stress.

Montgomery et al. (1983) proposed that reversible deacylation-reacylation of methylmalonyl-CoA may function as a shunt for the conversion of D to L-methylmalonyl-CoA in rats, working in parallel to the methylmalonyl-CoA epimerase (Montgomery, et al., 1983). In this proposed reaction, D-methylmalonyl-CoA is hydrolysed to the free acid and CoASH. Then the acid is randomly reesterified with CoASH producing a partly racemic mixture of D and L-methylmalonyl-CoA (Figure 2)

Figure 2: Proposed reaction mechanisms for the conversion of propionyl-CoA to succinyl-CoA. Figure adapted from Montgomery et al. (1983)

1) Propionyl-CoA carboxylase 2) Methylmalonyl-CoA Epimerase 3) Methylmalonyl-CoA Mutase 4) Acylase/Deacylase



One study showed that the inactivation of epimerase in HeLa cells by siRNA results in only a modest fall in [^{14}C]propionate incorporation (Dobson, et al., 2006). Another study showed that methylmalonyl-CoA can undergo non-enzymatic racemization by brief heating at 100 degrees at a neutral pH (Mazumder, et al., 1961) and that D-methylmalonyl-CoA can undergo slow non-enzymatic racemization *in vitro* at 30° (Mazumder, et al., 1962). Kuhnl et al (2005) characterized the phenotype of a *Caenorhabditis elegans mce-1* knockout mutant. The mutant showed a normal phenotype, revealing that methylmalonyl-CoA epimerase is not essential to normal worm development under laboratory conditions. Notably, under propionate stress conditions the *Caenorhabditis elegans mce-1* knockout mutant had an increased survival rate as compared to the wild-type. Kuhnl et al (2005) hypothesized that the reason for the increased survival is that in the absence of the epimerization reaction, the production of additional toxic metabolites or metabolic by-products derived from R-methylmalonyl-CoA is prevented. Another plausible explanation is that the accumulation of S-methylmalonyl-CoA protects against oxidative stress or prevents further excessive production of free radicals.

Other studies strengthen the argument that the epimerase is necessary for normal propionate metabolism. Mazumder et al (1962) found, using enzyme from sheep liver, that the formation of [^{14}C]succinyl-CoA was linearly related to the concentration of epimerase. Chandler et al (2006) created a *Caenorhabditis elegans mce-1* and an MCM deletion mutant. It was noted that the block in propionate metabolism observed in the *mce-1* mutant was as severe as that of the MCM mutant. In addition, the study demonstrated that if the free methylmalonic acid shunt, as described by Montgomery et

al. (1983), does in fact exist, the shunt is incapable of handling the normal flux through the pathway. Their results suggest that humans with *MCEE* defects will excrete methylmalonic acid and that epimerase-deficient cell lines will have a block in propionate incorporation. In addition, the high degree of conservation of the epimerase enzyme in eukaryotes, bacteria, and archaea (Kuhnl, et al., 2005) supports the hypothesis that it is essential for survival in eukaryotes, and that the gene product exists to play an important role in metabolism.

The above-mentioned studies showing the conflicting evidence regarding the necessity of the epimerase enzyme support the hypothesis that a defect in epimerase may be associated with moderately elevated methylmalonic acid levels. A deficiency of this enzyme may result in a continued, however lesser, degree of conversion of methylmalonyl-CoA to its product. As well, the clinical presentation and levels of methylmalonic acid excretion would be less severe in a case of epimerase deficiency than of MCM deficiency, if this condition indeed exists. As mentioned above, two cases of methylmalonic aciduria have recently been attributed to mutations affecting both alleles of the *MCEE* gene (Bikker, et al., 2006; Dobson, et al., 2006).

Rationale and Objectives of Study

Classical methylmalonic aciduria is an inborn error of metabolism known to result from defects in the enzyme methylmalonyl-CoA mutase (MCM) (*mut* complementation group) and from defects in the synthesis of the MCM cofactor adenosylcobalamin (*cblA, cblB, cblC, cblD, cblD* variant-*2/cblH*, and *cblF* groups). Historically there has been debate regarding the necessity of the enzyme methylmalonyl-CoA epimerase for normal propionate metabolism and regarding the association of defects in the epimerase with methylmalonic acid excretion. Studies on the epimerase enzyme have led us to the belief that a defective epimerase would cause a moderate clinical phenotype and excretion of methylmalonic acid. Recently, two patients who excreted methylmalonic acid were shown to have a homozygous nonsense mutation in the gene coding for methylmalonyl-CoA epimerase (*MCEE*).

The goal of this study was to investigate the frequency of mutations in *MCEE* in patients who excreted methylmalonic acid of unknown cause. Through these studies we set out to understand the contribution of the epimerase to methylmalonic acid excretion and whether or not a defective epimerase enzyme is associated with clinical symptoms. As well, we have set out to determine whether heterozygosity for mutations in the *MUT* gene, coding for MCM, was associated with elevated methylmalonic acid excretion in patients who excreted methylmalonic acid of unknown cause.

CHAPTER 2

Materials and Methods

2.1 Materials

Blood and Cell Culture DNA Mini Kit for extraction of genomic DNA as well as PCR reagents were purchased from QIAGEN Inc., Mississauga, ON; agarose from EMD, Gibbstown, NJ. PCR clean-up plates were from Millipore, Billerica, MA and sequencing reagents were purchased from Applied Biosystems, Foster City, CA. Tissue culture flasks (75 cm^2 and 175 cm^2) and 35 mm tissue culture dishes were purchased from Becton Dickenson, Palo Alto, CA. Cell culture medium was purchased from Invitrogen Canada Inc., Burlington, ON, the fetal bovine serum (F.B.S.) from Cansera International, Etobicoke, ON. [^{14}C]propionate was from GE Healthcare or New England Nuclear, Boston, MA. Unlabeled sodium propionate and the OHCbl were from Sigma, Oakville, ON. The [^{57}Co]CNCbl and the liquid scintillation cocktail were from MP Biomedicals, Orangeburg, NY and polyethylene glycol (PEG 1000) from J T Baker Inc, Phillipsburg, NJ. Both the trichloroacetic acid and sodium hydroxide were purchased from Fisher Scientific, Nepean, ON. The Gateway expression vector system, pENTR-TOPO directional cloning kit, and pLXSH retroviral vector containing Gateway cloning adapters were purchased from Invitrogen Canada Inc., Burlington, ON. The Pheonix Amphoteric cell line was a gift from G.P. Nolan, Stanford University.

2.2 Cell culture

All cell lines were stored at the Repository for Mutant Human Cells, Montreal Children's Hospital (Montreal, Quebec). Cell lines were determined to be free of

mycoplasma contamination by a modified method of the protocol used by Schneider et al. (1974). Cultures were incubated at 37°C in 5% CO_2 in 175 cm^2 flasks and were fed twice weekly with Minimum Essential Medium containing L-glutamine, Earle's salts, and non-essential amino acids. Medium was supplemented with 2.2 g/L sodium bicarbonate, 0.11 g/L sodium pyruvate, 10 mg/L ferric nitrate, and 1.5 g/L glucose.

2.3 Cell lines

Cell lines from 229 patients referred to the diagnostic laboratory at the Vitamin B_{12} Clinical Research Laboratory (Division of Medical Genetics, Department of Medicine, McGill University Health Centre) due to elevated levels of methylmalonic acid in blood and/or urine were studied. The clinical features of these patients varied; many had developmental delay, history of seizures, and hypotonia. Clinical findings were ascertained by written clinical reports at the time of referral. Their methylmalonic acid values were higher than those reported for healthy individuals but in most cases notably lower than in patients with known defects in methylmalonyl-CoA metabolism. At least one quantitative value for urinary or serum methylmalonic acid was available for 100 of these 229 patients and the values ranged from 8 to 1915 μmol/mmol creatinine (mean=279.7, n=74) and from 73 to 47,900 nmoles/L plasma (mean= 4894.3, n=37). Urine methylmalonic acid levels are dependent on age in the general population with a median value of 1.1 μmol/mmol creatinine in the infants under 30 days, 5.2 μmol/mmol creatinine in those 1 to 6 months, and 0.8 μmol/mmol creatinine in those 6 to12 months (Boulat, et al., 2003). Unrelated CEPH controls were used in the sequencing studies and control fibroblast lines MCH23 and MCH64 were used in biochemical studies.

Incorporation of label from [^{14}C]propionate into cellular macromolecules and synthesis of Cbl coenzyme derivatives from [^{57}Co]CNCbl was measured in all patient fibroblast lines. Somatic cell complementation analysis was performed on the fibroblast lines in which [^{14}C]propionate incorporation was sufficiently low to allow for interpretation of the effect of fusion. The initial propionate incorporation, complementation, and Cbl derivative experiments were performed by N. Matiazsuk, J. Lavallée, and D. Watkins and results permitted the division of the 229 cell lines into two groups. A subset of patients (n=30) with levels of [^{14}C]propionate incorporation that were below the control range were chosen for sequencing of both the *MUT* and *MCEE* genes. The balance of patients (n=199) were assigned to a second subset, and in this subset of patients with variable levels of propionate incorporation, only the *MCEE* gene was sequenced.

The first subset of patients (n=30) had incorporation of label from [^{14}C]propionate intermediate between that of controls and of patients with methylmalonic aciduria due to the *cblA*, *cblB,* and *mut* disorders (Table 1). [^{14}C]Propionate incorporation in patients was 3.2 ± 0.7 nmols propionate/mg protein/18h (mean ± SD) (control value 10.8 ± 3.7 nmols/mg protein/18h). [^{14}C]Propionate incorporation did not increase with addition of 3.75 μM OHCbl to the culture medium (3.6 ± 0.8 nmols propionate/mg protein/18h versus control at 10.9 ± 3.5 nmols/mg protein/18h). The synthesis of AdoCbl from [^{57}Co]CNCbl in patients was 12.7 ± 4.0 percent of total labelled cellular Cbl (control value 15 ± 4 percent). Somatic cell complementation was attempted on 22 of these patients, but in no case could they be assigned to any known complementation class. Characteristics of patient subset two is shown in Table 2.

Table 1 - Profile of patient subset one (n=30)

Age of onset refers to first noted clinical disease or to record of methylmalonic aciduria in asymptomatic cases. Propionate incorporation (Prop Inc) with and without the addition of hydroxycobalamin (OHCbl) measured in nmols/mg protein/18hr (control value 10.8 ± 3.7 and 10.9 ± 3.5 respectively). The synthesis of adenosylcobalamin (AdoCbl) expressed as percentage of total cellular cobalamin following incubation of fibroblasts in 25 pg/ml of [^{57}Co]CNCbl for 96 hours (control value of AdoCbl is 15% ± 4 of total cellular Cbl).

Cell line	Sex	Ethnicity	Age of Onset	Prop Inc -OHCbl	Prop Inc +OHCbl	AdoCbl%
WG1485	Female	White	6 months	2.7	2.9	14.6
WG2021	Male	White/ Asian	Infancy	3.6	4.3	21.5
WG2022	Female	White/ Asian	Infancy	3.5	4.1	22.3
WG2084	Female	White	Birth	2.7	2.7	13.7
WG2096	Female	Black	Birth	1.6	2.7	15.9
WG2388	Male	White	2 years	4	4.3	11.9
WG2393	Female	White	4 months	3.8	4.2	10.2
WG2428	Female	Native American	4 months	2.8	2.8	8.3
WG2436	Female	Not Available	6 months	2.7	2.8	10
WG2480	Female	Not Available	Birth	3.6	3.5	15.5
WG2481	Male	White	6 months	1.8	1.7	9.6
WG2575	Male	White	4 months	4.1	4.5	11.2
WG2686	Male	White	7 months	3.3	4.3	19.9
WG2695	Female	Native American/ Black/ Hispanic	6 months	3.4	3.7	12.2
WG2716	Male	White	Birth	3.2	3.1	14
WG2718	Female	Phillipino	1 year	3	3.4	9.1
WG2727	Female	White	1 month	3.2	3.1	14.8
WG2797	Male	White	Asymptomatic	3.7	3.5	12.4
WG2823	Female	Hispanic	3 months	3.2	3.1	13.2
WG2837	Male	White	Asymptomatic	3.2	3.4	14.2
WG3012	Female	White	4 months	2.4	4.2	10.8
WG3013	Male	Pakistani	11 months	3.8	4.6	7.5
WG3086	Male	White	8 months	3.4	3.3	9
WG3092	Male	White	2 months	4.2	4.2	18.1
WG3099	Female	Not Available	3 months	3	3.1	11.5
WG3108	Female	Not Available	14 months	3.1	3.4	15.3
WG3186	Male	White	3 years	3.3	3.9	7.8
WG3187	Female	White	14 months	2.4	2.5	10.5
WG3221	Female	White	6 weeks	3.7	5.3	9.9
WG3384	Female	White	5 weeks	4.6	4.5	7.5

Table 2 - Biochemical presentation of patient subset two (n=199).

Values for propionate incorporation (Prop) without and with the addition of hydroxycobalamin (OHCbl) measured in nmols/mg protein/18hr (control value 10.8 ± 3.7 and 10.9 ± 3.5 respectively). The synthesis of adenosylcobalamin (AdoCbl) expressed as percentage of total cellular cobalamin following incubation of fibroblasts in 25 pg/ml of [^{57}Co]CNCbl for 96 hours (control value of AdoCbl is 15% ± 4 of total cellular Cbl).

Cell Line	Prop (w/out OHCbl)	Prop (w/ OHCbl)	AdoCbl %
WG1075	5.2	6.1	16.0
WG1246	11.4	11.0	13.6
WG1255	8.2	7.6	14.7
WG1274	3.9	4.4	8.7
WG1377	4.7	4.0	20.7
WG1385	5.7	5.8	13.9
WG1424	10.3	9.7	15.9
WG1454	18.0	17.0	12.3
WG1473	8.3	8.6	14.7
WG1476	14.4	15.1	15.9
WG1484	7.9	7.9	7.8
WG1534	15.5	15.4	33.3
WG1649	6.1	6.7	12.8
WG1702	6.9	8.0	21.0
WG1704	8.0	9.9	16.2
WG1739	4.9	5.1	18.0
WG1754	12.0	13.0	21.6
WG1755	7.8	8.8	20.1
WG1778	9.0	10.0	17.5
WG1808	12.0	13.0	16.4
WG1832	6.0	8.0	13.8
WG1865	3.4	5.0	10.2
WG1878	6.4	8.4	13.7
WG1942	5.8	5.9	14.4
WG1946	8.3	8.8	17.7
WG1962	5.6	8.3	17.9
WG1970	5.3	6.5	10.7
WG2001	7.5	8.5	18.1
WG2017	8.8	11.7	14.7

Cell Line	Prop (w/out OHCbl)	Prop (w/ OHCbl)	AdoCbl %
WG2042	8.9	8.0	17.6
WG2050	10.0	15.0	17.4
WG2053	9.5	9.2	15.4
WG2054	8.1	8.4	15.4
WG2075	8.0	9.0	19.7
WG2097	8.0	9.8	16.6
WG2098	7.0	8.2	15.2
WG2126	4.0	5.0	11.1
WG2140	7.8	7.2	13.3
WG2141	10.2	10.8	15.7
WG2145	15.0	14.0	16.2
WG2146	8.0	8.0	14.4
WG2151	10.0	8.0	12.2
WG2156	8.0	7.0	16.9
WG2158	7.2	6.2	15.5
WG2162	8.9	8.0	15.3
WG2166	5.8	6.9	16.1
WG2197	8.7	8.2	13.5
WG2204	9.6	6.5	17.5
WG2218	5.8	5.0	16.7
WG2229	6.0	7.2	13.6
WG2237	7.7	7.0	13.3
WG2246	7.6	7.8	18.5
WG2248	6.1	5.6	17.6
WG2252	5.8	5.9	15.8
WG2253	6.9	6.5	13.5
WG2257	5.6	6.0	12.4
WG2264	3.7	3.9	11.1
WG2270	5.8	5.5	17.9

Cell Line	Prop (w/out OHCbl)	Prop (w/ OHCbl)	AdoCbl %
WG2281	4.4	4.4	14.1
WG2286	11.0	11.0	18.2
WG2316	5.0	5.4	11.3
WG2344	6.0	6.0	13.3
WG2361	5.5	5.6	11.9
WG2363	6.5	6.7	12.8
WG2368	4.8	6.0	13.5
WG2376	5.5	5.6	13.3
WG2380	6.6	7.8	9.9
WG2387	8.0	8.7	16.3
WG2389	5.5	6.2	9.5
WG2394	4.5	6.1	8.6
WG2412	6.0	7.7	17.3
WG2427	5.0	5.0	18.2
WG2441	6.0	7.0	13.0
WG2442	9.0	9.0	14.5
WG2463	7.0	7.0	15.3
WG2482	5.9	5.3	9.4
WG2484	6.8	6.4	12.2
WG2494	4.3	4.4	7.4
WG2497	4.7	4.5	7.3
WG2502	7.2	7.0	9.9
WG2554	9.0	11.0	12.6
WG2558	8.0	9.0	12.5
WG2570	4.8	4.9	14.1
WG2585	7.0	7.4	14.8
WG2592	11.0	11.0	14.8
WG2594	8.0	8.0	12.7
WG2598	4.7	5.4	12.4
WG2626	8.0	8.0	14.0
WG2645	5.5	6.4	12.8
WG2648	5.8	6.7	12.3
WG2649	7.8	8.3	15.1
WG2657	6.7	7.4	13.3
WG2678	4.2	5.2	17.0
WG2701	5.2	5.7	16.5

Cell Line	Prop (w/out OHCbl)	Prop (w/ OHCbl)	AdoCbl %
WG2721	17.5	19.8	10.9
WG2731	4.2	4.4	11.2
WG2740	5.5	5.5	11.0
WG2766	5.4	5.4	13.2
WG2767	6.0	6.3	17.1
WG2771	8.0	8.4	14.4
WG2790	10.7	10.6	17.2
WG2791	9.1	10.6	14.0
WG2792	10.8	10.9	14.7
WG2802	5.3	6.0	11.7
WG2809	6.2	6.5	16.7
WG2810	6.0	7.2	14.1
WG2819	6.5	6.3	18.4
WG2824	6.2	6.6	15.9
WG2828	7.4	7.1	10.0
WG2854	5.3	5.6	10.0
WG2855	5.1	6.2	7.4
WG2856	6.7	6.9	16.2
WG2861	6.0	6.1	14.4
WG2862	6.5	7.5	10.0
WG2874	6.0	6.0	12.4
WG2879	7.0	7.1	10.8
WG2881	5.6	5.3	20.1
WG2885	8.1	8.5	15.5
WG2892	5.6	5.7	16.5
WG2928	5.2	6.0	23.8
WG2946	4.5	4.8	11.2
WG2955	4.8	5.0	15.5
WG2961	12.0	12.0	13.3
WG2964	7.3	8.0	14.6
WG2979	3.6	4.3	14.5
WG2981	3.4	5.0	12.0
WG2984	5.8	6.3	8.8
WG2997	7.8	9.5	14.2
WG3002	6.8	7.7	13.8
WG3005	8.2	8.0	15.8

Cell Line	Prop (w/out OHCbl)	Prop (w/ OHCbl)	AdoCbl %
WG3006	6.4	6.3	18.2
WG3034	9.5	11.2	8.8
WG3037	5.7	5.6	15.8
WG3047	4.6	7.4	14.8
WG3048	6.3	6.5	5.6
WG3057	8.1	9.6	13.9
WG3075	8.0	8.7	15.6
WG3085	5.0	5.5	11.3
WG3125	5.3	5.1	11.5
WG3132	6.4	7.0	15.2
WG3133	8.5	10.0	14.6
WG3147	5.7	6.2	11.7
WG3149	4.9	4.9	12.2
WG3155	5.6	6.0	12.6
WG3162	5.3	5.9	14.2
WG3167	5.5	5.9	15.4
WG3171	8.3	8.9	14.6
WG3193	6.8	7.5	10.6
WG3204	3.8	3.5	10.0
WG3212	6.7	6.9	13.5
WG3234	5.5	6.9	8.5
WG3235	3.6	4.0	12.1
WG3236	5.2	5.8	11.3
WG3237	3.9	4.3	12.5
WG3241	6.0	7.1	15.1
WG3245	8.3	10.0	10.7
WG3252	6.0	6.4	9.4
WG3253	6.1	8.0	11.2
WG3256	7.6	9.0	12.0
WG3258	6.7	8.3	11.5
WG3259	5.8	7.1	8.3
WG3260	6.9	8.0	9.5
WG3264	10.2	10.7	9.9
WG3270	9.6	10.5	13.5
WG3273	13.0	16.2	10.0

Cell Line	Prop (w/out OHCbl)	Prop (w/ OHCbl)	AdoCbl %
WG3284	8.0	8.5	14.5
WG3291	3.9	4.8	10.5
WG3292	8.1	8.3	13.9
WG3303	6.9	8.2	10.5
WG3306	4.1	5.0	10.2
WG3320	10.7	11.5	9.9
WG3321	12.3	13.1	13.4
WG3331	7.1	7.6	12.8
WG3341	8.4	9.0	13.3
WG3348	6.0	6.4	9.6
WG3349	6.3	7.7	9.6
WG3350	8.9	11.3	12.8
WG3351	4.1	4.1	9.1
WG3353	3.6	3.8	12.1
WG3357	4.8	6.0	9.2
WG3360	7.8	11.4	13.4
WG3363	5.3	6.4	8.6
WG3375	5.8	6.1	16.8
WG3379	9.4	11.4	17.3
WG3381	7.5	9.0	11.0
WG3386	6.7	8.2	11.9
WG3393	4.0	4.9	9.8
WG3394	7.2	11.0	9.3
WG3398	8.4	11.3	13.7
WG3418	5.7	5.9	10.2
WG3434	7.6	8.5	16.0
WG3440	10.7	13.0	10.4
WG3444	6.6	7.3	12.5
WG3449	10.6	13.3	12.1
WG3450	7.6	8.1	18.3
WG3455	10.5	12.1	9.3
WG3457	10.4	13.0	10.8
WG3462	14.4	16.4	12.9
WG3492	6.2	9.9	10.0

2.4 Mutation detection

Analysis of the *MCEE* gene was performed on genomic DNA from all 229 patients. Analysis of the *MUT* gene was also performed on genomic DNA from the first subset of patients (n=30). Fibroblasts from each cell line were grown to confluence in tissue culture flasks. Cells were removed from the flasks by trypsinization, pelleted by centrifugation, and washed with phosphate buffered saline (PBS). Cell pellets were stored at –80°C until DNA extraction.

2.4.1 Amplification of exons from genomic DNA

The three *MCEE* exons (RefSeq NM_032601.2) and the 13 *MUT* exons (RefSeq NM_000255.1) (including noncoding exon 1) and surrounding regions were amplified by PCR. All reported mutations were resequenced from fresh PCR product. The *MCEE* gene was screened in 80 unrelated CEPH controls and the coding exons of the *MUT* gene were screened in at least 40 controls. *MCEE* primers were designed using the Primer3 electronic resource program (Rozen and Skaletsky, 2000), and *MUT* primers were previously described by Worgan et al. (2006). The optimized *MCEE* PCR conditions are listed in Table 3.

PCR was performed using Qiagen protocols and contained 0.2 µl of HotStarTaq, 5 of µl buffer, 0.5 µl of primer, 2 µl of $MgCl_2$, 1 µl of DNTPs, 39 µl of DNA grade water, and 2 µl of DNA per reaction. The thermocycler program used were as follows:

1) 96.0°C for 12 min
2) 96.0°C for 30 sec
3) Annealing temperature for 1 min
4) 72.0°C for 1 min
5) Steps 2-4 repeated 35 times
6) 72.0°C for 10 min

Table 3 - Optimized primer conditions for the *MCEE* and *MUT* genes

***MUT* Exon**	**Forward primer 5' - 3'**	**Reverse Primer 5' - 3'**	**Annealing Temperature in degrees Celsius**
1	CTGGCTGTGTGTGGATGTCTGA	TAAAGGACAGAGCGGGAGAG	59
2	TCCCACCCCCTCTTCTAAT	ACAGAGATTAACCCCCAAAAA	55
3	CATTTTACCTTGATTCCAGACTCTT	CCAGATTCCTGCAAGTAACGA	58
4	CCAAGGTTTGGGTCTACAAC	AAAATGGTCCTATGCATTTC	55
5	TTTCTTCCATATGCATAAAACTGT	GTGCCACATTGCTCAGAAAA	57
6	TTGCTGTTTAATCATGTTGCTG	TCTGAAAACAAAGTTGCAAAGTG	58
7	TTCTCCCAAGACTTAAGAGGTTTT	ACATATGCTTGCCTGTGTGC	58
8	CCCCTTCTCAGATTGGGATT	TTAGCCAGAGCCCAGAACAC	58
9	TGCATCAGGGTTCTAATCTCTTG	TTGGGCTCACATGGTTTACA	58
10	GATGCATAAAGGCATCCAGAA	TCAAGGGGATTGTGCTAACAG	59
11	ACTTGAAAGATTTGCTGTG	TGTCTGTCATCATCATTTTACTAC	48
12	CAGGGTTTTTATAGTCATTA	CAAGATTCCCATCACAGT	50
13	CCGTGAAAATGGAAATAGTGG	GCATGACACCAGGCCTATAA	58
***MCEE* Exon**			
1	CCAAACGCCTTTCAACTCTC	AGGCAATCCCCGCTACTAAG	62
2	TCTAACTGCAGCTCAGAATTAGC	CCTGTTACATGGGAGGTCAAAC	62
3	TGCATGAGCTCTCAAAGTTTTT	TTTCTGTAATTCAGTCTTTAACTGTGA	55

PCR products were run on an agarose gels to check for the DNA product of the appropriate exon size.

2.4.2 Sequencing of exons

PCR products were purified and the sequencing reactions were performed in 96-well plates in 10 µl volume, consisting of 2 µl PCR product, 0.5 µl of primer, 1.75 µl of 5× sequencing buffer, 0.5 µl of BigDye Terminator Cycle Sequencing Version 3.1, and 5.25 µl of water. The sequencing reaction was performed under the following conditions:

1) 96.0°C for 30 sec
2) 50.0°C for 10 sec
3) 60.0°C for 4 min
4) Steps 1-3 were repeated 25 times

After completion of the sequencing reaction, products were prepared for sequencing analysis. 3 µl of 3M sodium acetate, 64.6 µl of 100% ethanol, and 22.4 µl of H_2O were

added to each sample and the sequencing plate was incubated for 15 minutes. The precipitated products were then pelleted by centrifugation at 3000×g for 30 minutes. Then products were washed using 100 µl of 70% ethanol. Ethanol was then removed and samples were centrifuged at 700×g for 30 seconds. Remaining ethanol was evaporated and the pellet was resuspended in 10 µl of formamide. Products were analyzed on an ABI 3730 DNA analyzer. Sequences were processed using sequence analysis software and were assembled for analysis using the Phred/Phrap/Consed system (Ewing, et al., 1998; Gordon, et al., 1998). All exons were sequenced in one direction and a standardized mutation nomenclature was used (den Dunnen and Antonarakis, 2000). In this nomenclature the 1+ position is the A of the ATG translation initiation codon.

2.5 [^{14}C]Propionate incorporation

Cell lines were plated at a density of 400,000 cells per 35 mm tissue culture dish. After plating, cells were incubated for 18 hours in Puck's F medium supplemented with 15% fetal bovine serum (Cansera International) and 100 µmol/L [1-[^{14}C]propionate] (New England Nuclear or GE Healthcare) diluted with unlabeled propionate to give a final specific activity of 10 µCi/µmol. Cultures were then washed three times with PBS and cellular macromolecules were precipitated by three incubations with 5% trichloroacetic acid at 5°. The precipitated material was dissolved in 0.2N sodium hydroxide and radioactivity was determined by liquid scintillation counting. Protein levels were determined using the method of Lowry (Lowry, et al., 1951). [^{14}C]Propionate incorporation values are reported in nmols propionate/mg protein/18h.

2.6 Complementation analysis

Equal numbers of two cell lines were plated in 35 mm tissue culture dishes for a total density of 400,000 cells per dish. After cells had plated, half of the cultures were fused by exposure for 1 minute to 40% (w/v) polyethylene glycol in PBS. Cultures were washed thoroughly to remove PEG. [^{14}C]Propionate incorporation was then assessed in parallel fused and unfused cultures to determine whether correction of the biochemical defect had occurred. Statistical significance was determined using the Student's t-test.

2.7 Transfection of wild-type *MCEE* gene

Wild-type *MCEE* cDNA was generated by RT-PCR (using polydT primers) from total RNA extracted from a control human fibroblast line. Identity of this product was confirmed by sequence analysis. The cDNA was cloned into a Gateway expression vector system using the pENTR-TOPO directional cloning kit, and then was further subcloned into a pLXSH retroviral vector (Miller and Buttimore, 1986) containing Gateway cloning adapters. The retroviral vector containing cDNA was transiently infected into a Phoenix Amphoteric cell line using the HEPES-buffered saline/ $Ca_3(PO_4)_2$ protocol (http://www.stanford.edu/group/nolan/retroviral_systems/retsys.html). Fibroblasts with mutations at the *MCEE* locus and controls were each infected by exposure to virus-containing medium with polybrene (4 μg/ml). Cells were then incubated in 200 U/ml of hygromycin containing media to select for cells that had incorporated the vector. [^{14}C]propionate incorporation was then performed on transfected and untransfected cell lines as described above.

CHAPTER 3

Results

3.1 Mutation analysis

Sequence changes in the *MCEE* gene were identified in five patients. Two patients with decreased [^{14}C]propionate incorporation (WG2084 and WG2797) were homozygous for a nonsense mutation (c.139C>T, p.R47X) in exon 2 (Figure 3). In the second patient subset, one patient (WG2098) was homozygous for a novel missense mutation (c.178A>C, p.K60Q) in the second exon, and two patients (WG1476 and WG3270) were heterozygous for a novel missense mutation in exon 3 (c.427C>T, p.R143C) (Figures 4 and 5). None of these sequence changes were seen in 80 controls. One CEPH control was heterozygous for a novel sequence change in the *MCEE* gene (c.166C>T, p.P56S). The distribution of mutations in *MCEE* is shown in figure 6. Further biochemical and clinical data on patients found to have mutations in *MCEE* is described in Table 4.

Analysis of the *MUT* gene in the first patient subset revealed one patient (WG2823) to be heterozygous for a novel missense mutation in exon 6 (c.1091A>C, p.Y364S) and two patients (WG2021 and WG3186) to be heterozygous for a novel silent base change in exon 3 (c.711A>G, p.P237P) (Figures 7 and 8). None of these sequence changes were seen in 40 controls. A number of polymorphisms were detected in patients and controls in both the *MCEE* and *MUT* genes in approximately equal proportions (Tables 5 and 6).

Figure 3

Homozygous nonsense mutation in *MCEE* exon 2 at position c.139C>T, p.R47X

Top row: WG2084, homozygous C>T. Middle row: WG2797, homozygous C>T. Bottom row: control, homozygous wild-type.

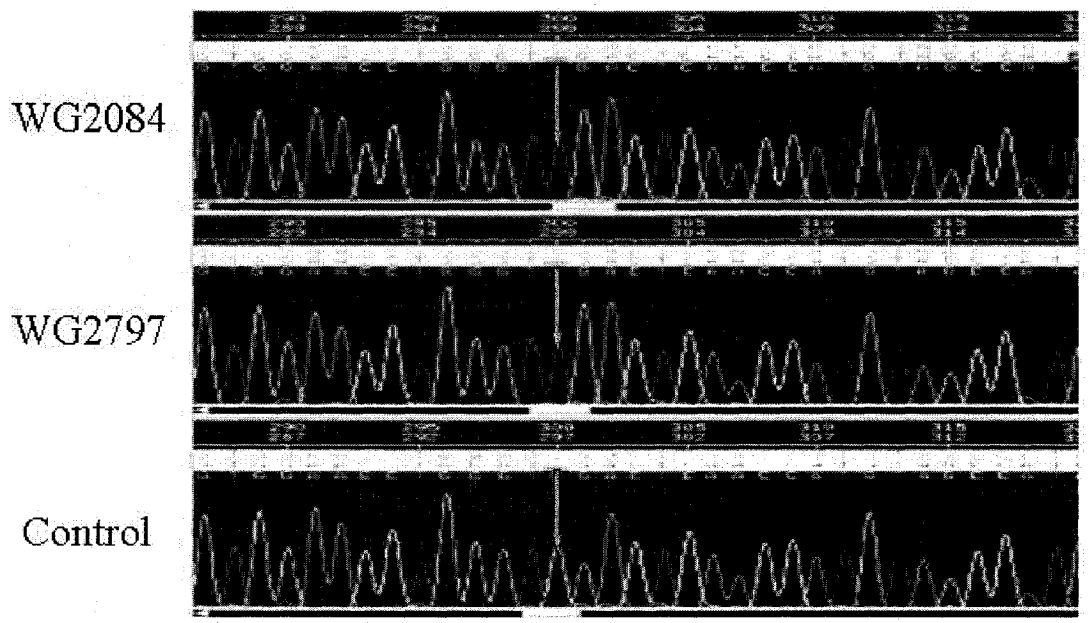


Figure 4

Homozygous missense mutation in *MCEE* exon 2 at position c.178A>C, p.K60Q

Top row: WG2098, homozygous A>C. Bottom row: control, homozygous wild-type.

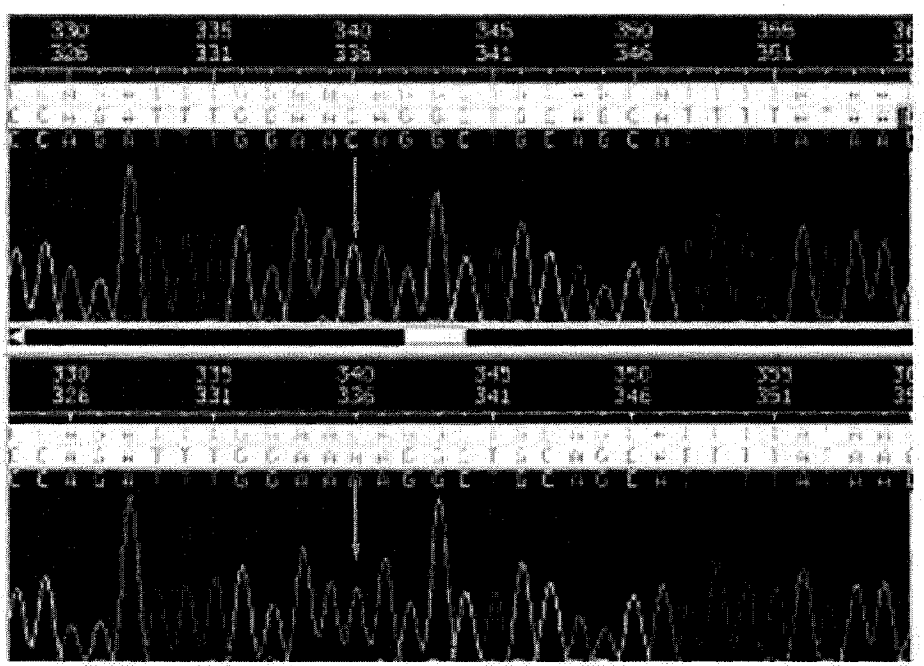


Figure 5

Heterozygous missense mutation in *MCEE* exon 3 at position c.427C>T, p.R143C

Top row: WG1476, heterozygous C>T. Middle row: WG3270, heterozygous C>T. Bottom row: control, homozygous wild-type.

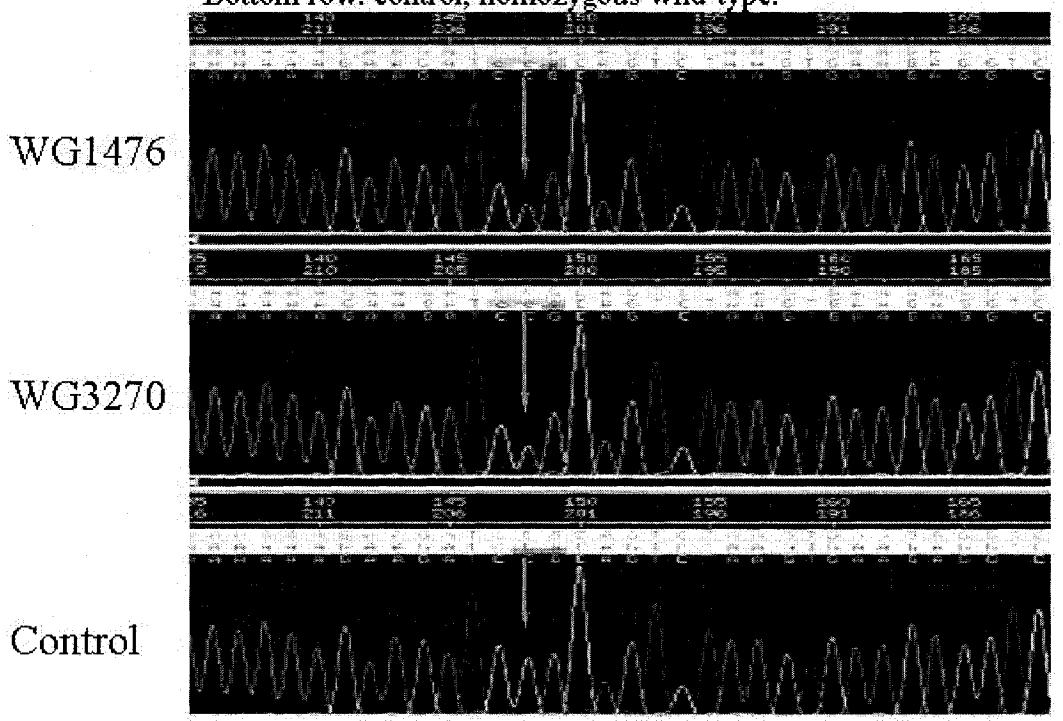
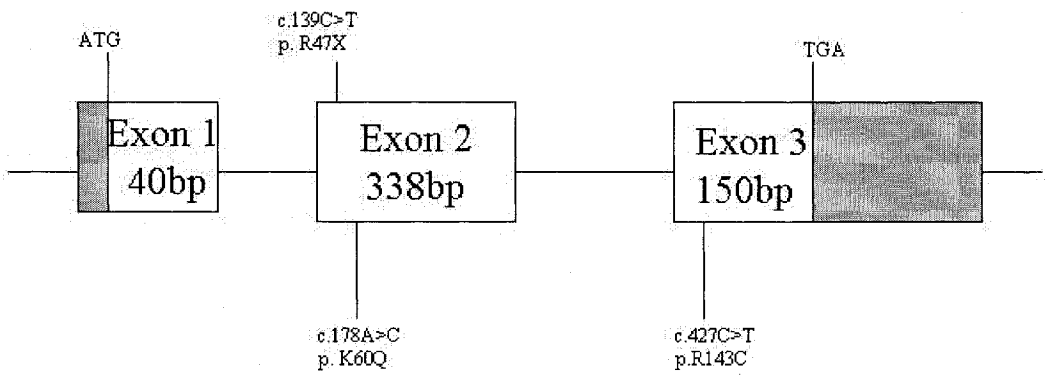


Figure 6

Distribution of mutations identified in the *MCEE* gene



The gene structure according to the reference sequence NM_032601.2 is shown.
The nonsense mutation is shown above the plane of the gene and missense mutations below.
Diagram not to scale. bp = base pairs.

Figure 7

Heterozygous missense mutation *MUT* exon 6 at position c.1091A>C, p.Y364S

Top row: WG2823, heterozygous A>C. Bottom row: control, homozygous wild-type.

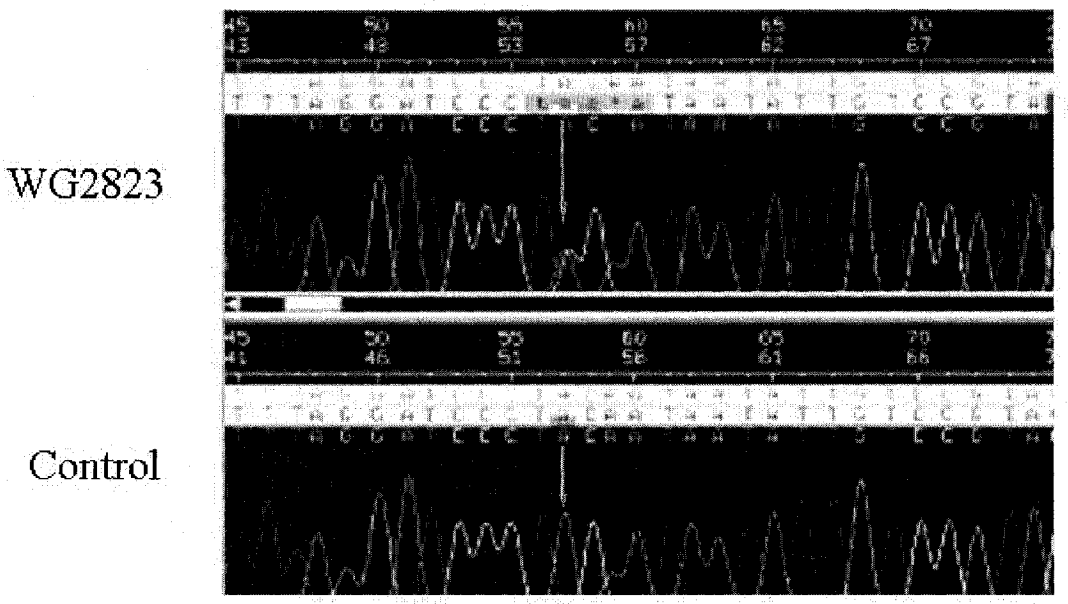


Figure 8

Heterozygous silent mutation in *MUT* exon 3 at position c.711A>G, p.P237P

Top row: WG2021, heterozygous A>G. Middle row: WG3186, heterozygous A>G. Bottom row: control, homozygous wild-type.

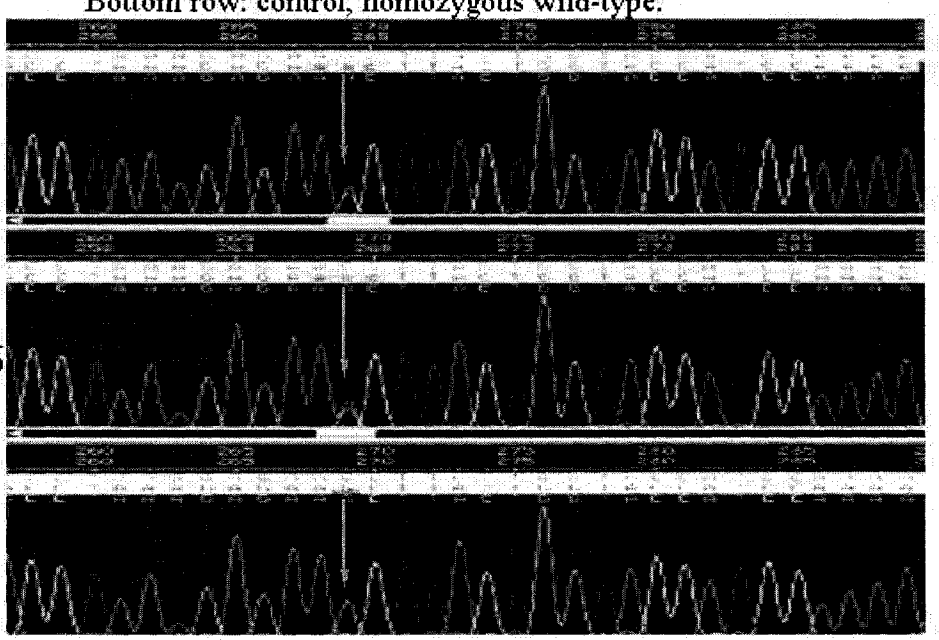


Table 4 - Clinical and biochemical data for patients with mutations in the *MCEE* gene. Age of onset refers to first noted clinical disease or to record of methylmalonic aciduria in the asymptomatic cases. Values for propionate incorporation (Prop Incorp) without and with the addition of hydroxycobalamin (OHCbl) measured in nmols/mg protein/18hr (control value 10.8 ± 3.7 and 10.9 ± 3.5 respectively). The synthesis of adenosylcobalamin (AdoCbl) expressed as percentage of total cellular cobalamin following incubation of fibroblasts in 25 pg/ml of [^{57}Co]CNCbl for 96 hours (control value of AdoCbl is 15% ± 4 total cellular cobalamin). MMA = methylmalonic acid.

Cell Line	Mutation	Presentation	Age of Onset	Prop Incorp w/o OHCbl	Prop Incorp w OHCbl	AdoCbl%	MMA Values
WG 2084 (Bikker, et al, 2006)	*MCEE* Homozygous c.139C>T, p.R47X	- sepiapterin reductase deficiency - normal intellect - slow motor development - ataxia - hypotonia	birth	2.7	2.7	13.7	1175 µmol/mmol creatinine
WG 2797	*MCEE* Homozygous c.139C>T, p.R47X	- clinically normal - affected sibling with mma	asymptomatic	3.9	4.0	12.4	1400 µmol/mmol creatinine
WG 2098	*MCEE* Homozygous c.178A>C, p.K60Q	- ataxia - deteriorated motor function - normal MRI - dysarthria - mild spastic paraparesis	3 years	7.0	8.2	12.7	621 µmol/mmol creatinine
WG 1476	*MCEE* Heterozygous c.427C>T, p.R143C	- mma found on newborn screen - persistent mma - clinically normal	asymptomatic	14.4	15.1	15.9	58 µmol/mmol creatinine
WG 3270	*MCEE* Heterozygous c.427C>T, p.R143C	- excessive sleeping - seizures - normal development	10 weeks	8.0	8.3	13.5	6820 nmoles/L serum

Table 5 - Common polymorphisms detected in the coding and surrounding regions of the *MCEE* gene. The genotype of patients and controls is described for each common polymorphism.

SNP number	Locus	Patients	Controls
rs2012479	c.-68G>T	GG: 34/250=0.14	GG: 9/80=0.11
		GT: 108/250=0.43	GT: 39/80=0.49
		TT: 108/250=0.43	TT: 32/80=0.40
rs11126319	c.40+115G>T	GG: 245/250=0.98	GG: 80/80=1.00
		GT: 5/250=0.02	GT: 0/80=0.00
		TT: 0/250=0.00	TT: 0/80=0.00
*	c.102C>G, p.P34P	CC: 246/250=0.98	CC: 75/80=0.94
		CG: 4/259=0.02	CG: 5/80=0.06
		GG: 0/250=0.00	GG: 0/80=0.00
rs11541017	c.227C>T, p.A76V	CC: 121/250=0.48	CC: 36/80=0.45
		CT: 100/250=0.40	CT: 35/80=0.44
		TT: 29/250=0.12	TT: 9/80=0.11
rs6748672	c.331G>T, p.L104R	GG: 171/250=0.68	GG: 49/80=0.61
		GT: 69/250=0.28	GT: 27/80=0.34
		TT: 10/250=0.04	TT: 4/80=0.05

* Sequence variation not listed in SNP database, however, present in control lines.

Table 6 - Common polymorphisms detected in the coding and surrounding regions of the *MUT* gene. The genotype of patients and controls is described for each common polymorphism.

SNP number	Locus	Patients	Controls
rs2229384	c.636A>G, p.K212K	AA: 14/30=0.47	AA: 18/47=0.38
		AG: 13/30=0.43	AG: 22/47=0.47
		GG: 3/30=0.10	GG: 7/47=0.15
rs9369901	c.912-110C>T	CC: 14/30=0.47	CC: 28/47=0.60
		CT: 15/30=0.50	CT: 16/47=0.34
		TT: 1/30=0.03	TT: 3/47=0.06
rs4715130	c.1333-185	AA: 13/30=0.43	AA: 21/48=0.44
		AG: 11/30=0.37	AG: 19/48=0.40
		GG: 6/30=0.20	GG: 8/48=0.17
rs17851388	c.1495G>A, p.A499T	GG: 22/30=0.73	GG: 33/46=0.72
		AG: 7/30=0.23	AG: 13/46=0.28
		AA: 1/30=0.03	AA: 0/46=0.00
rs9473558	c.1595G>A, p.R532H	GG: 13/30=0.43	GG: 21/47=0.45
		GA: 11/30=0.37	GA: 18/47=0.38
		AA: 6/30=0.20	AA: 8/47=0.17
rs9473557	c.1677-153	AA: 13/30=0.43	AA: 20/47=0.43
		AG: 11/30=0.37	AG: 19/47=0.40
		GG: 6/30=0.20	GG: 8/47=0.17
rs6458687	c.2011G>A, p.V671I	GG: 14/30=0.47	GG: 18/48=0.38
		GA: 13/30=0.43	GA: 23/48=0.48
		AA: 3/30=0.10	AA: 7/48=0.15

3.2 Complementation analysis

Two lines homozygous for the c.139C>T, p.R47X nonsense mutation in *MCEE* were analyzed. WG2278 was identified previously by Dobson et al. (2006) and WG2084 was identified simultaneously by our group and by Bikker et al. (2006). [^{14}C]Propionate incorporation was not increased significantly in mixed fused cultures of WG2278 and WG2084 compared to mixed unfused cultures of the two cell lines (p=0.7799) (Figure 9). WG2278 complemented the genetic defect in *mut* (p=0.0022), and *cblB* fibroblasts (p=0.0023). This patient has shown variable complementation with fibroblasts from the *cblA* group and was initially considered to be an atypical member of this complementation class (Watkins, et al., 2000). Fibroblasts from WG2084 complemented the genetic defect in *mut* (p=0.001), *cblA* (p=0.0003), and *cblB* (p=0.0001) fibroblasts.

3.3 Transfection experiments

Fibroblasts from WG2278, WG2084, a *cblC* cell line (WG3568), and a control cell line (MCH64) were transfected with the *MCEE* construct. The [^{14}C]propionate incorporation in fibroblasts from WG2278 and WG2084 was significantly increased in transfected compared to untransfected cells (p=0.0002 and 0.0041, respectively). In the *cblC* fibroblast cell line transfection with the *MCEE* construct had no effect on [^{14}C]propionate incorporation (p=0.429). The [^{14}C]propionate incorporation decreased slightly in the control fibroblast cell line with added construct (p=0.0425) (Figure 10).

Figure 9: Complementation analysis of *MCEE* deficient fibroblasts. Both patients (WG2278 and WG2084) harbour the c.139C>T, p.R47X homozygous nonsense mutation in *MCEE*. Fibroblasts from patients WG2278 and WG2084 were both fused with a *mut* cell line (WG3572) and with each other. [^{14}C]Propionate incorporation was measured in parallel in fused and unfused cultures. Increased [^{14}C]propionate incorporation observed in fusions with *mut* line but not between lines with epimerase mutations. Error bars represent standard deviation from triplicates of the complementation assay. Figure represents the results of one experiment done in triplicate however the experiments were repeated with similar results.

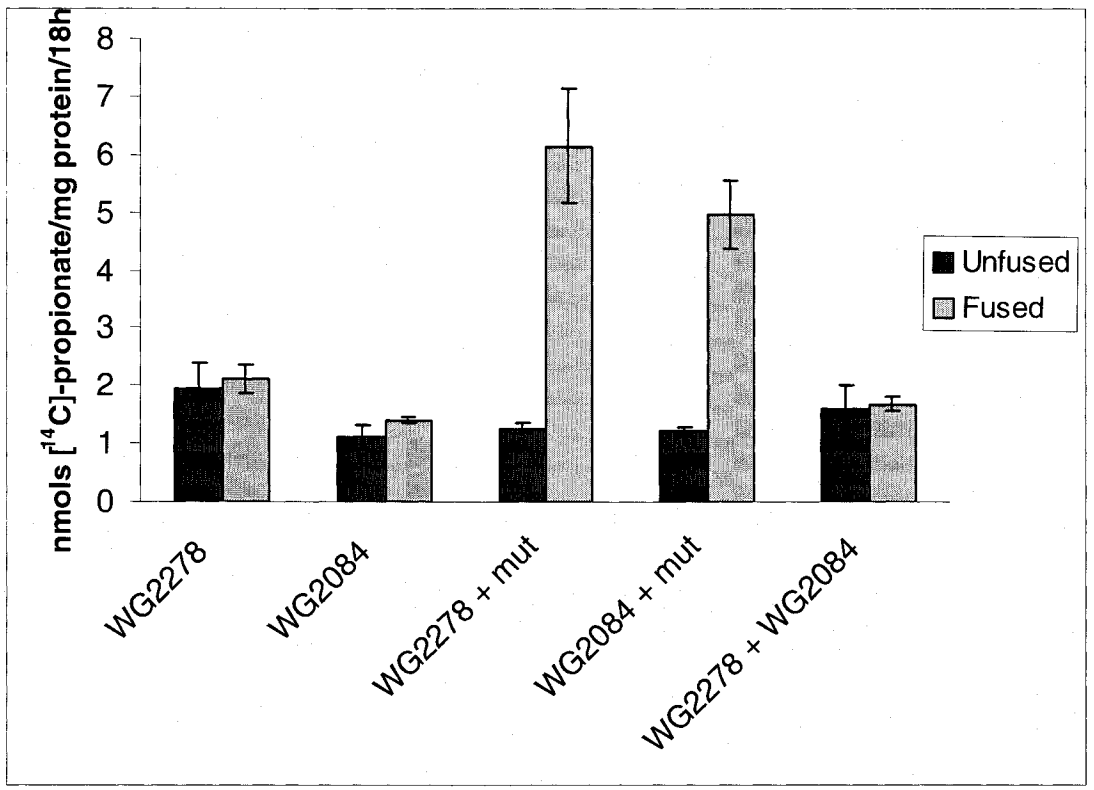
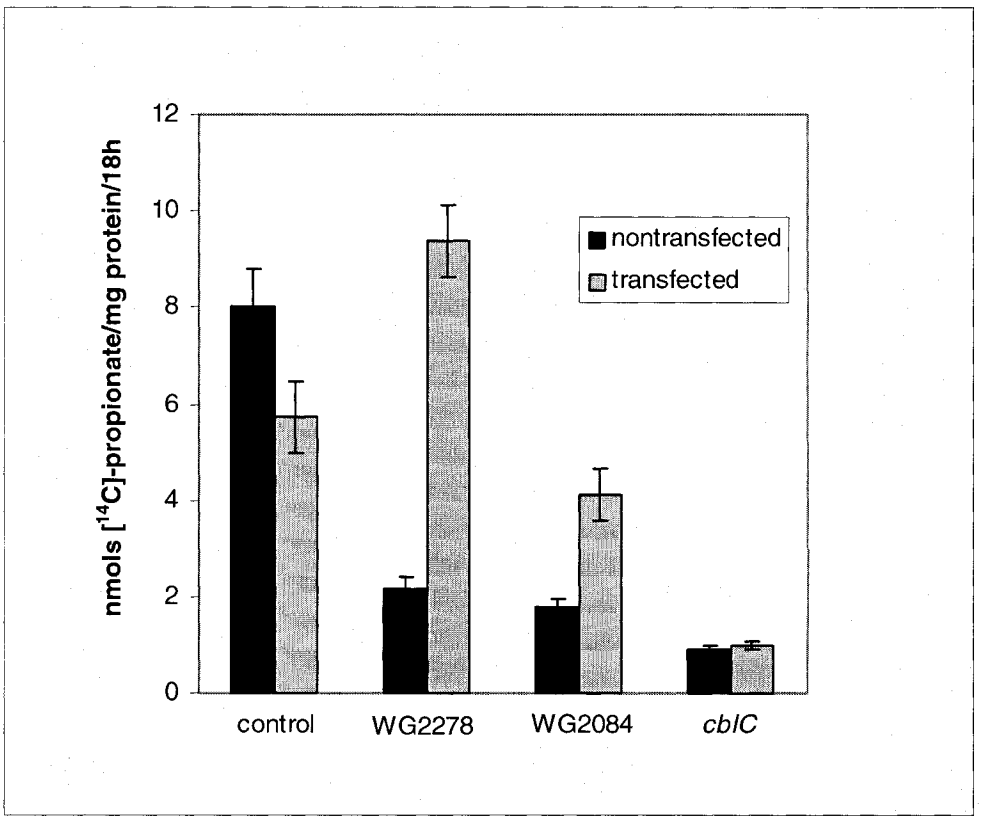


Figure 10: [^{14}C]Propionate incorporation results. [^{14}C]Propionate incorporation in cultures of control cell line (MCH64), epimerase deficient WG2278 and WG2084, and *cblC* cell line (WG3568) with and without transfected insert of wild-type *MCEE* cDNA. Error bars represent standard deviation from triplicates of the propionate incorporation assay. Figure represents the results of one experiment done in triplicate however the experiments were repeated with similar results.



CHAPTER 4

Discussion

This project was undertaken to investigate the frequency of mutations in the *MCEE* gene in patients with moderate methylmalonic aciduria of unknown cause. Interest in the role of mutations in *MCEE* arose with the identification of a patient (WG2278) with methylmalonic aciduria of unknown etiology that was homozygous for a nonsense mutation (c.139C>T, p.R47X) in this gene (Dobson, et al., 2006). In the present study, *MCEE* mutations were identified in five additional patients. Two patients (WG2084 and WG2797), both with decreased [^{14}C]propionate incorporation, were homozygous for the previously described c.139C>T, p.R47X mutation [The mutation in one of these patients, WG2084, was simultaneously identified by Bikker et al (2006)]. In the second subset of patients one patient (WG2098) was found to be homozygous for a missense mutation (c.178A>C, p.K60Q) at a lysine residue that is conserved in epimerase from mammals and nematodes (Kuhnl, et al., 2005). Two other patients from the second subset (WG1476 and WG3270) were heterozygous for a missense mutation affecting codon 143 of the *MCEE* gene (c.427C>T, p.R143C). This represents a nonconservative change at an arginine that is conserved in animals, some archaea, and some bacteria (Kuhnl, et al., 2005). One CEPH control was heterozygous for a sequence change in the *MCEE* gene that was not seen in any of the patients screened in this study and that has not been previously described in the literature.

The reduced [^{14}C]propionate incorporation in two cell lines (WG2278 and WG2084) with the homozygous nonsense mutation was complemented by fusion with *cblA*, *cblB,* and *mut* fibroblasts but not by fusion with each other. Therefore, the

decreased [^{14}C]propionate incorporation in these patients is likely the result of decreased epimerase function. This is further supported by the results of the transfection studies; overexpression of wild-type epimerase in these cell lines corrected the cellular phenotype. An additional patient who excreted methylmalonic acid was homozygous for a missense mutation affecting a well-conserved amino acid (c.178A>C, p.K60Q). This mutation was not seen in 80 unrelated CEPH controls, supporting its pathogenicity. It is not clear whether the heterozygous mutations in *MCEE* seen in patients WG1476 and WG3270 are the cause of their methylmalonic aciduria. It is noteworthy that there was a large discrepancy in methylmalonic acid values in the two heterozygotes; WG1476 had a value of 58 μmoles methylmalonic acid/mmol creatinine while WG3270 had a much higher value of 6820 nmoles/L serum. It is possible that there is an undetected mutation in *MCEE* in trans or that polymorphisms in and around *MCEE* affect the expression of epimerase in these patients. Perhaps synergistic heterozygosity for mutations in different genes in the methylmalonate metabolism pathway are responsible for the mild methylmalonic acid excretion (Vockley, et al., 2000).

As previously mentioned, there is currently debate over whether epimerase activity is necessary for normal cellular function. On the one hand, studies have suggested that an alternate non-enzymatic route for the conversion of the D to the L isomer exists in parallel to epimerase, and it has been argued that non-enzymatic conversion might be sufficient to maintain methylmalonyl-CoA metabolism in the absence of a functional epimerase. These studies suggest that a defect in the epimerase enzyme may be associated with only a partial block in the conversion of methylmalonyl-CoA to succinyl-CoA, and with only a mild elevation of methylmalonic acid that may not

give rise to any clinical consequences (asymptomatic methylmalonic aciduria). It is possible that mutations in the *MCEE* gene act as genetic modifiers, increasing the susceptibility of individuals to environmental differences, and resulting in increased methylmalonic acid excretion. However, methylmalonyl-CoA epimerase is present in a wide range of organisms including both eukaryotes and prokaryotes, with more than one sixth of the residues completely conserved over a large phylogenetic distance (Kuhnl, et al., 2005), suggesting that this gene is actively conserved through evolution. Conservation of the epimerase enzyme supports the alternate hypothesis that it is essential for survival in eukaryotes.

The cause of the mild methylmalonic acid excretion in the patients in which mutations were not identified is currently unknown. Perhaps some of the patients presented with transient methylmalonic acid excretion, not attributable to a structural defect in any of the genes implicated in methylmalonyl-CoA metabolism. There is also the possibility that mutations outside the coding region of the *MCEE* gene that influence expression or that create alternative splice sites may play a role. Another possible explanation for moderate methylmalonic acid excretion is heterozygosity for mutations in other genes that effect metabolism of methylmalonyl-CoA. Screening of the coding regions of the *MUT* gene in 30 patients (subset one) with mild methylmalonic aciduria and moderately reduced [^{14}C]propionate incorporation revealed a novel missense mutation in one patient (WG2823) in the heterozygous state at a residue that is conserved among mammals. This suggests that heterozygosity for mutations at *MUT* may contribute to mild methylmalonic acid excretion in some patients.

Two patients (WG2021 and WG3186) were heterozygous for a silent *MUT* mutation (c.711A>G, p.P237P). Studies have shown that silent mutations or polymorphisms can affect the function of a protein (Kimchi-Sarfaty, et al., 2007), RNA stability, and may alter splice sites. Therefore these changes may cause disease even though the nucleotide sequence remains unaltered. It is unclear whether the silent mutation is causal of disease in these cases but the sibling of WG2021 (WG2022) also presented with methylmalonic aciduria, and had decreased [^{14}C]propionate incorporation by fibroblasts, but did not share the *MUT* mutation. Both siblings have recently been shown to be heterozygous for a mutation in the *SUCLA2* gene, which encodes succinyl-CoA synthase (Carlo Dionisi-Vici, and personal communication). Succinyl-CoA synthase deficiency has recently been identified as a cause of methylmalonic aciduria (Carrozzo, et al., 2007; Ostergaard, et al., 2007). The finding of a mutation in each sibling at the *SUCLA2* locus suggests that the *MUT* sequence change does not contribute to the methylmalonic acid excretion seen in WG2021. Perhaps synergistic heterozygosity for mutations in different genes in the methylmalonate metabolism pathway are causal of the presentation of patient WG2021 who is heterozygous for sequence variations in both the *MUT* and *SUCLA2* genes.

The results of the current study suggest that mutations in *MCEE* can be associated with increased excretion of methylmalonic acid, but are not a common cause. Including results presented in this current work, four individuals (three families) have now been described that are homozygous for the same c.139C>T, p.R47X nonsense mutation in the *MCEE* gene (Dobson et al., 2006; Bikker et al., 2006) and methylmalonic acid excretion has been detected in each of these individuals. To the best of our knowledge these three

families are unrelated. Results of complementation analysis and transfection experiments show that methylmalonic aciduria in these cases is due to decreased epimerase activity. However, it is not clear that the methylmalonic acid excretion in these patients is consistently associated with clinical illness; if it is, there appears to be a large range in phenotype. The patient WG2278, reported by Dobson et al. (2006) had severe metabolic acidosis, but her sibling, as well as one of the patients reported here (WG2797) were clinically asymptomatic despite being homozygous for the same nonsense mutation. The serious clinical findings in patient WG2084 seem all to be associated with this patient's sepiapterin reductase deficiency (Bikker et al., 2006). The patient homozygous for the c.178A>C, p.K60Q missense mutation (WG2098) had a clinical picture that included ataxia, deteriorated motor function, dysarthria, mild spastic paraparesis, and a reportedly normal MRI. The apparent lack of a distinct clinical presentation in patients with homozygous *MCEE* mutations is inconsistent with the strong conservation of the gene across a large phylogenetic distance; one-sixth of the residues are identical in epimerases from eukaryotes, bacteria and archaea (Kuhnl et al., 2005), suggesting strong selection for the gene. The reason for this contradiction is not known.

Summary and Conclusion

In summary, genomic DNA was extracted from cell lines with mild methylmalonic acid excretion of unknown origin and the *MCEE* gene was analyzed through PCR and sequencing of individual exons. Complementation analysis and transfection experiments were performed to further characterize the effect of mutations found in *MCEE*. As well, we have analyzed the *MUT* gene in a subset of patients with moderate methylmalonic acid excretion as well as moderately decreased [^{14}C]propionate incorporation.

Our findings have improved the understanding of the frequency of *MCEE* mutations in patients who excrete methylmalonic acid and of the association of defects in epimerase with clinical symptoms. Our findings also contribute further to the debate over the requirement of the epimerase for cellular metabolism. We have shown that five patients with mild methylmalonic aciduria have mutations in the *MCEE* gene, and it seems likely that these mutations would be related to their methylmalonic acid excretion. As well, two cell lines with the same homozygous nonsense mutation in the epimerase did not display correction in [^{14}C]propionate incorporation toward control values after fusion, indicating that the two lines had the same defect in the metabolism of methylmalonate. Furthermore, transfection of *MCEE* cDNA into two cell lines with the same homozygous nonsense mutation resulted in correction of the cellular defect, indicating that mutations in the epimerase gene were causal of the observed phenotype. Overall, we conclude that a functional epimerase enzyme is required for normal methylmalonate metabolism, and that defects at this locus can result in, but are not a frequent cause of the excretion of methylmalonic acid. Epimerase defects do not appear

to cause a distinct group of clinical symptoms. As well, the finding of a heterozygous missense mutation in *MUT* in one patient suggests that heterozygous changes in this gene may contribute to methylmalonic acid excretion.

Claims to Originality

The following are original contributions to the knowledge on methylmalonic aciduria:

1) Determination of the frequency of mutations in *MCEE* in a large cohort of patients who excrete methylmalonic acid of unknown cause. As well, identification of novel mutations in *MCEE* in this cohort of patients.

2) Evidence that the fusion of cells from two fibroblast lines with the same homozygous nonsense mutation in *MCEE* did not correct the defect in propionate metabolism; therefore indicating that the two lines have the same factor causal of decreased propionate incorporation (a defective epimerase enzyme).

3) Evidence that transfection of *MCEE* cDNA into two cell lines with the same homozygous nonsense mutation in *MCEE* resulted in correction towards a normal phenotype, indicating that a defect in the epimerase gene was causal of the observed phenotype.

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Appendix A

List of Publications and Presentations

Original Publications:

1) **Gradinger AB**, Belair C, Worgan LC, Li CD, Roquis D, Lavallée J, Matiaszuk N, Watkins D, Rosenblatt DS. The molecular basis of atypical methylmalonic aciduria: Search for mutations in the methylmalonyl-CoA epimerase gene (*MCEE*). Accepted by *Hum Mutat.*

2) Dobson CM, **Gradinger A**, Longo N, Wu X, Leclerc D, Lerner-Ellis J, Lemieux M, Belair C, Watkins D, Rosenblatt DS, Gravel RA. 2006. 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.* 88: 327-333.

3) Lerner-Ellis JP, **Gradinger AB**, Watkins D, Tirone JC, Villeneuve A, Dobson CM, Montpetit A, Lepage P, Gravel RA, Rosenblatt DS. 2006. Mutation and biochemical analysis of patients belonging to the *cblB* complementation class of vitamin B_{12}-dependent methylmalonic aciduria. *Mol Genet Metab.* 87: 219-225.

Published Abstracts:

1) **Gradinger AB**, Worgan LC, Lerner-Ellis JP, Watkins D, Moras E, Roquis C, Dore C, and Rosenblatt DS. The molecular basis of atypical methylmalonic aciduria: Search for mutations in the methylmalonyl-CoA mutase and epimerase genes. American Society of Human Genetics (A.S.H.G.) Annual Meeting. Salt Lake City, Utah. October 25-29, 2005. *Am J Hum Genet.* Abstract 1453. Page 274.

Presentations (Oral and Poster):

1) The molecular basis of atypical methylmalonic aciduria: Search for mutations in the methylmalonyl-CoA mutase and epimerase genes. Poster presentation, Federation of American Societies for Experimental Biology (F.A.S.E.B.) Conference on Folic Acid Vitamin B_{12} and One-Carbon Metabolism. Palm Springs, California. August 5-10, 2006.

2) The molecular basis of atypical methylmalonic aciduria. Oral project presentation for the McGill University Department of Human Genetics, faculty and students. Montreal, Quebec. May 25, 2006.

3) The molecular basis of atypical methylmalonic aciduria: Search for mutations in the methylmalonyl-CoA mutase and epimerase genes. Poster presentation, Gordon Conference on Cobalamin & Corphins. Oxford, England. September 18-23, 2005.

4) The molecular basis of atypical methylmalonic aciduria: Search for mutations in the methylmalonyl-CoA mutase and epimerase genes. Oral project presentation for the Canadian Institute of Health Research (C.I.H.R.) vitamin B_{12} collaborator group. Montreal, Quebec. April 6, 2005.

Abstract B

Ethics Approval and Certificates

Appendix C

First Authoured Publication,

Official Letter of Journal Acceptance, and Contribution of Authours

ABSTRACT

Methylmalonic aciduria is known to result from defects in the enzyme methylmalonyl CoA mutase (MCM) (*mut* complementation group) and from defects in the synthesis of the MCM cofactor adenosylcobalamin (*cblA*, *cblB*, *cblC*, *cblD*, and *cblF* groups). Two patients who excrete methylmalonic acid have recently been shown to have a homozygous nonsense mutation in the gene coding for methylmalonyl CoA epimerase (*MCEE*). To further understand the cause of methylmalonic acid excretion, the *MCEE* gene was sequenced in 229 patients with elevations of methylmalonic acid excretion for which no cause was known. Mutations in *MCEE* were detected in five patients: two patients homozygous for c.139C>T, p.R47X, one patient homozygous for c.178A>C, p.K60Q, and two patients heterozygous for c.427C>T, p.R143C. Fusion of fibroblast lines from two patients homozygous for c.139C>T, p.R47X did not result in correction of [^{14}C]propionate incorporation toward control values while the defect in these fibroblasts was complemented by *mut*, *cblA*, and *cblB* fibroblasts. Infection with wild-type *MCEE* cDNA resulted in correction of the biochemical phenotype in cells from both patients.

KEY WORDS: methylmalonic aciduria; methylmalonyl CoA epimerase; MCEE; methylmalonyl CoA mutase; MUT

INTRODUCTION

Isolated methylmalonic aciduria is known to occur due to defects in MCM (*mut* complementation group) or to inborn errors in the metabolism of cobalamin that result in decreased synthesis of AdoCbl, namely the *cblA, cblB*, and *cblD variant 2* complementation groups (Fenton et al., 2001; Suormala et al., 2004). Isolated methylmalonic aciduria has recently been associated with succinyl CoA synthase deficiency (Carrozzo et al., 2007; Ostergaard et al., 2007). The *mut* complementation group accounts for the largest number of patients with isolated methylmalonic aciduria. At least 178 mutations in the *MUT* gene on chromosome 6p21 have been identified in *mut* patients (Worgan et al., 2006; Lempp et al., 2007). Mutations in the *MMAA* gene on 4q31.1-31.2 and in the *MMAB* gene on chromosome 12q24 have been identified in patients with the *cblA* and *cblB* disorders respectively (Lerner-Ellis et al., 2004; Lerner-Ellis et al., 2006). However, a number of patients have been identified who excrete elevated levels of methylmalonic acid who could not be assigned to any complementation group.

Historically, there has been debate over the physiological necessity of the methylmalonyl CoA epimerase enzyme (Montgomery et al., 1983), and *in vitro* studies have shown that racemization of methylmalonyl CoA can proceed in the absence of a functional epimerase (Mazumder et al.,1961; Mazumder et al.,1962). The epimerase gene (*MCEE*) (MIM 608419) on chromosome 2p13.3 was the first cobalamin related gene to be identified on the basis of prokaryotic gene arrangements (Bobik et al., 2001). Recently, two cases of methylmalonic aciduria have been attributed to homozygosity for a nonsense mutation of the *MCEE* gene (Dobson et al., 2006; Bikker et al., 2006). These are the first confirmed cases of epimerase deficiency (MIM 251120) in humans; the subject of an earlier report thought to have an epimerase defect (Kang et al., 1972) was later shown to have mutase deficiency (Rosenberg and Scriver, 1980).

In this study the *MCEE* gene was sequenced in 229 patients who excreted elevated levels of methylmalonic acid and who could not be assigned to any class of defect known to cause methylmalonic aciduria. We have performed complementation analysis and transduction experiments to further characterize the effect of mutations in *MCEE*. As well, we have analyzed the *MUT* gene in a subset of patients with moderate

methylmalonic acid excretion as well as moderately decreased [^{14}C]propionate incorporation, an indirect measure of cellular MCM function.

MATERIALS AND METHODS

Patients and Controls

Cell lines from 229 patients referred to the diagnostic laboratory at the Vitamin B_{12} Clinical Research Laboratory (Division of Medical Genetics, Department of Medicine, McGill University Health Centre) due to elevated levels of methylmalonic acid in blood or urine were studied. Clinical findings were ascertained by written clinical reports at the time of referral. These varied among patients; many had developmental delay, a history of seizures, and hypotonia. Their methylmalonic acid values were higher than those reported for healthy individuals but in most cases notably lower than in patients with known defects in methylmalonyl CoA metabolism. At least one quantitative value for urinary or serum methylmalonic acid was available for 100 of these 229 patients and the values ranged from 8 to 1915 μmol/mmol creatinine (urine) (mean=279.7, n=74) and from 73 to 47,900 nmoles/L (plasma) (mean= 4894.3, n=37). Methylmalonic acid levels are dependent on age in the general population with a median value of 1.1 μmol/mmol creatinine in the infants under 30 days, 5.2 μmol/mmol creatinine in those 1 to 6 months, and 0.8 μmol/mmol creatinine in those 6 to12 months (Boulat et al., 2003). Unrelated CEPH controls were used in the sequencing studies and control fibroblast lines MCH23 and MCH64 were used in biochemical testing. This study was approved by the Research Ethics Board of the Royal Victoria Hospital, Montreal, Quebec, Canada. Incorporation of label from [^{14}C]propionate into cellular macromolecules (a measure of MCM activity) and synthesis of cobalamin coenzyme derivatives from [^{57}Co]CNCbl were measured in all patient fibroblast lines (Watkins et al., 2000).

Mutation Detection

Genomic DNA was extracted from patient fibroblasts, sequenced and analyzed using previously described techniques (Lerner-Ellis et al., 2006). The three *MCEE* exons (RefSeq NM_032601.2) and the 13 *MUT* exons (RefSeq NM_000255.1) (including noncoding exon 1) were amplified. All reported mutations were resequenced from fresh PCR product. The *MCEE* gene was sequenced in 80 unrelated CEPH controls and the coding exons of the *MUT* gene were sequenced in at least 40 unrelated CEPH controls. *MCEE* primers were designed using the Primer3 program (Rozen and Skaletsky, 2000) (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), and *MUT* primers were previously described by Worgan et al. (2006). The optimized *MCEE* PCR conditions are listed in Supplemental Table 1. All exons were sequenced in one direction. The mutation nomenclature used was that recommended by den Dunnen and Antonarakis (2000) (http://www.hgvs.org/mutnomen/). In this method the 1+ position is the A of the ATG translation initiation codon.

Supplemental Table 1 - Optimized *MCEE* primer conditions.

Amplicon	Forward primer 5'-3'	Reverse primer 5'-3'	Annealing temperature
Exon 1	CCAAACGCCTTTCAACTCTC	AGGCAATCCCCGCTACTAAG	62
Exon 2	TCTAACTGCAGCTCAGAATTAGC	CCTGTTACATGGGAGGTCAAAC	62
Exon 3	TGCATGAGCTCTCAAAGTTTTT	TTTCTGTAATTCAGTCTTTAACTGTGA	55

[^{14}C]Propionate Incorporation and Complementation Analysis

[^{14}C]Propionate incorporation and complementation analysis were carried out as previously described (Watkins et al., 2000). Statistical significance was determined using Student's t-test.

Transduction of the Wild-Type *MCEE* Gene

Wild-type *MCEE* cDNA was generated by RT-PCR (using polydT primers) from total RNA extracted from a control human fibroblast line. Identity of this product was confirmed by sequence analysis. The cDNA was cloned into a Gateway expression vector system (Invitrogen) using the pENTR-TOPO directional cloning kit, and then was further subcloned into a pLXSH retroviral vector (Miller and Buttimore, 1986) containing Gateway cloning adapters (Invitrogen). The retroviral vector containing cDNA was transiently transfected into a Phoenix Amphoteric cell line (gift from G.P. Nolan, Stanford University) using the HEPES-buffered saline/$Ca_3(PO_4)_2$ protocol (http://www.stanford.edu/group/nolan/retroviral_systems/retsys.html). Fibroblasts with mutations at the *MCEE* locus and controls were each infected by exposure to virus-containing medium with polybrene (4 μg/ml). Cells were then incubated in medium containing 200 U/ml of hygromycin to select for cells that had incorporated the vector. [^{14}C]Propionate incorporation was then performed on infected and uninfected cell lines.

RESULTS

Biochemical Studies

The 229 cell lines were divided into two groups. A subset of patients (n=30) had incorporation of label from [^{14}C]propionate intermediate between that of controls and patients with methylmalonic aciduria due to the *cblA*, *cblB,* or *mut* disorders (Table 1). Incorporation in patients was 3.2 ± 0.7 nmols propionate/mg protein/18h (control value 10.8 ± 3.7 nmols/mg protein/18h). [^{14}C]Propionate incorporation did not increase with addition of 3.75μM hydroxy-cobalamin to the culture medium (3.6 ± 0.8 nmols propionate/mg protein/18h versus control at 10.9 ± 3.5 nmols/mg protein/18h). The synthesis of AdoCbl from [^{57}Co]CNCbl in patients was 12.7 ± 4.0 percent of total cellular cobalamin (control value 15 ± 4 percent). Somatic cell complementation was attempted on 22 of these patients, but in no case could they be assigned to any known complementation class.

Table 1- Profile of patient subset #1 (n=30) with reduced [^{14}C]propionate incorporation and who excreted methylmalonic acid. Age of onset refers to first noted clinical disease or to record of methylmalonic aciduria in the asymptomatic cases. Propionate incorporation (Prop Inc) with and without the addition of hydroxycobalamin (OHCbl) measured in nmols/mg protein/18hr. The synthesis of adenosylcobalamin (AdoCbl) from [^{57}Co]CNCbl expressed as percentage of total cellular cobalamin.

Cell line	Sex	Ethnicity	Age of Onset	Prop Inc -OHCbl	Prop Inc +OHCbl	AdoCbl%
WG1485	Female	White	6 months	2.7	2.9	14.6
WG2021	Male	White/ Asian	Infancy	3.6	4.3	21.5
WG2022	Female	White/ Asian	Infancy	3.5	4.1	22.3
WG2084*	Female	White	Birth	2.7	2.7	13.7
WG2096	Female	Black	Birth	1.6	2.7	15.9
WG2388	Male	White	2 years	4	4.3	11.9
WG2393	Female	White	4 months	3.8	4.2	10.2
WG2428	Female	Native American	4 months	2.8	2.8	8.3
WG2436	Female	Not Available	6 months	2.7	2.8	10
WG2480	Female	Not Available	Birth	3.6	3.5	15.5
WG2481	Male	White	6 months	1.8	1.7	9.6
WG2575	Male	White	4 months	4.1	4.5	11.2
WG2686	Male	White	7 months	3.3	4.3	19.9
WG2695	Female	Native American/ Black/ Hispanic	6 months	3.4	3.7	12.2
WG2716	Male	White	Birth	3.2	3.1	14
WG2718	Female	Phillipino	1 year	3	3.4	9.1
WG2727	Female	White	1 month	3.2	3.1	14.8
WG2797*	Male	White	Asymptomatic	3.7	3.5	12.4
WG2823	Female	Hispanic	3 months	3.2	3.1	13.2
WG2837	Male	White	Asymptomatic	3.2	3.4	14.2
WG3012	Female	White	4 months	2.4	4.2	10.8
WG3013	Male	Pakistani	11 months	3.8	4.6	7.5
WG3086	Male	White	8 months	3.4	3.3	9
WG3092	Male	White	2 months	4.2	4.2	18.1
WG3099	Female	Not Available	3 months	3	3.1	11.5
WG3108	Female	Not Available	14 months	3.1	3.4	15.3
WG3186	Male	White	3 years	3.3	3.9	7.8
WG3187	Female	White	14 months	2.4	2.5	10.5
WG3221	Female	White	6 weeks	3.7	5.3	9.9
WG3384	Female	White	5 weeks	4.6	4.5	7.5

*denotes patients with the homozygous nonsense mutation c.139C>T, p.R47X.

The second subset of patients (n=199) showed levels of both [^{14}C]propionate incorporation (7.2 ± 2.5 nmols propionate/mg protein/18h) and synthesis of AdoCbl (13.6 ± 3.4 percent of total cellular cobalamin) that were generally within the control range. Complementation analysis could not be performed due to the relatively normal levels of [^{14}C]propionate incorporation.

Mutation Analysis

Sequence changes in the *MCEE* gene were identified in five patients, including two patients with reduced [^{14}C]propionate incorporation and three patients with normal [^{14}C]propionate incorporation. Two patients with decreased [^{14}C]propionate incorporation (WG2797 and WG2084) were homozygous for a nonsense mutation (c.139C>T, p.R47X) in exon 2. Among the 199 patients with normal [^{14}C]propionate incorporation, one patient (WG2098) was homozygous for a novel missense mutation (c.178A>C, p.K60Q) in the second exon, and two patients (WG1476 and WG3270) were heterozygous for a novel missense mutation in exon 3 (c.427C>T, p.R143C). None of these sequence changes was seen in 80 controls. One CEPH control was heterozygous for a novel sequence change in the *MCEE* gene (c.166C>T, p.P56S).

Analysis of the *MUT* gene in the 30 patients with decreased [^{14}C]propionate incorporation revealed one patient (WG2823) to be heterozygous for a novel missense mutation in exon 6 (c.1091A>C, p.Y364S) and two patients (WG2021 and WG3186) to be heterozygous for a novel silent base change in exon 3 (c.711A>G, p.P237P). None of these sequence changes was seen in CEPH controls.

A number of polymorphisms were detected in patients and controls in both the *MCEE* and *MUT* genes. The frequency of the polymorphisms was similar in patients and controls. Further biochemical and clinical data on patients found to have mutations in *MCEE* is described in Table 2.

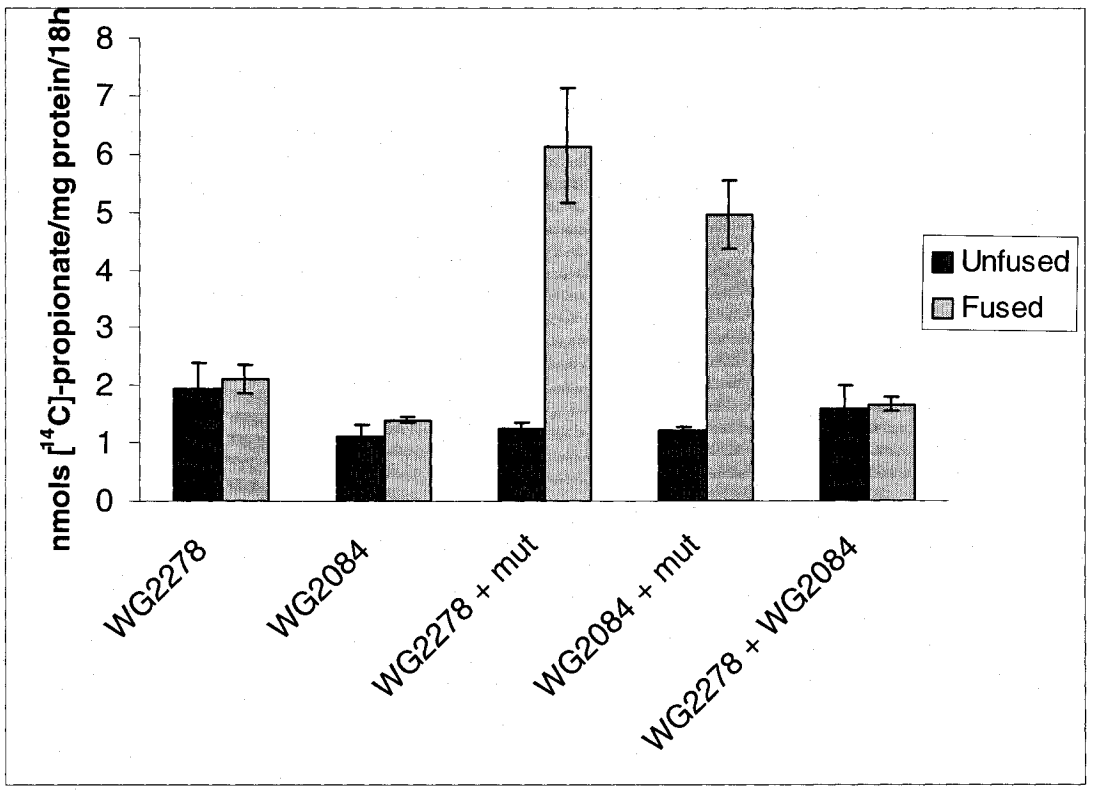
Table 2- Clinical and biochemical data for patients with mutations in the *MCEE* gene. Age of onset refers to first noted clinical disease or to record of methylmalonic aciduria in the asymptomatic cases. Propionate incorporation (Prop Inc) with and without the addition of hydroxycobalamin (OHCbl) measured in nmols/mg protein/18hr. The synthesis of adenosylcobalamin (AdoCbl) from [^{57}Co]CNCbl expressed as percentage of total cellular cobalamin. MMA =methylmalonic acid.

Cell Line	Mutation	Presentation	Age of Onset	Prop Incorp w/o OHCbl	Prop Incorp w OHCbl	AdoCbl%	MMA Values
WG 2084 (Bikker, et al, 2006)	*MCEE* Homozygous c.139C>T, p.R47X	- sepiapterin reductase deficiency - normal intellect - slow motor development - ataxia - hypotonia	birth	2.7	2.7	13.7	1175 µmol/mmol creatinine
WG 2797	*MCEE* Homozygous c.139C>T, p.R47X	- clinically normal - affected sibling with mma	asymptomatic	3.9	4.0	12.4	1400 µmol/mmol creatinine
WG 2098	*MCEE* Homozygous c.178A>C, p.K60Q	- ataxia - deteriorated motor function - normal MRI - dysarthria - mild spastic paraparesis	3 years	7.0	8.2	12.7	621 µmol/mmol creatinine
WG 1476	*MCEE* Heterozygous c.427C>T, p.R143C	- mma found on newborn screen - persistent mma - clinically normal	asymptomatic	14.4	15.1	15.9	58 µmol/mmol creatinine
WG 3270	*MCEE* Heterozygous c.427C>T, p.R143C	- excessive sleeping - seizures - normal development	10 weeks	8.0	8.3	13.5	6820 nmoles/L serum

Complementation Analysis

Two lines homozygous for the c.139C>T, p.R47X nonsense mutation in *MCEE* were analyzed. WG2278 was identified previously by Dobson et al. (2006) and WG2084 was identified simultaneously by our group and by Bikker et al. (2006). [^{14}C]Propionate incorporation was not increased significantly in mixed fused cultures of WG2278 and WG2084 compared to mixed unfused cultures of the two cell lines (p=0.7799) (Figure 1). *Mut* and *cblB* fibroblasts complemented the genetic defect in WG2278 (p=0.0022 and p=0.0023 respectively). This patient had shown variable complementation with fibroblasts from the *cblA* group and was initially considered to be an atypical member of this complementation class (Watkins et al., 2000). *Mut*, *cblA*, and *cblB* fibroblasts complemented the genetic defect in WG2084 (p=0.001, p=0.0003, and p=0.0001 respectively).

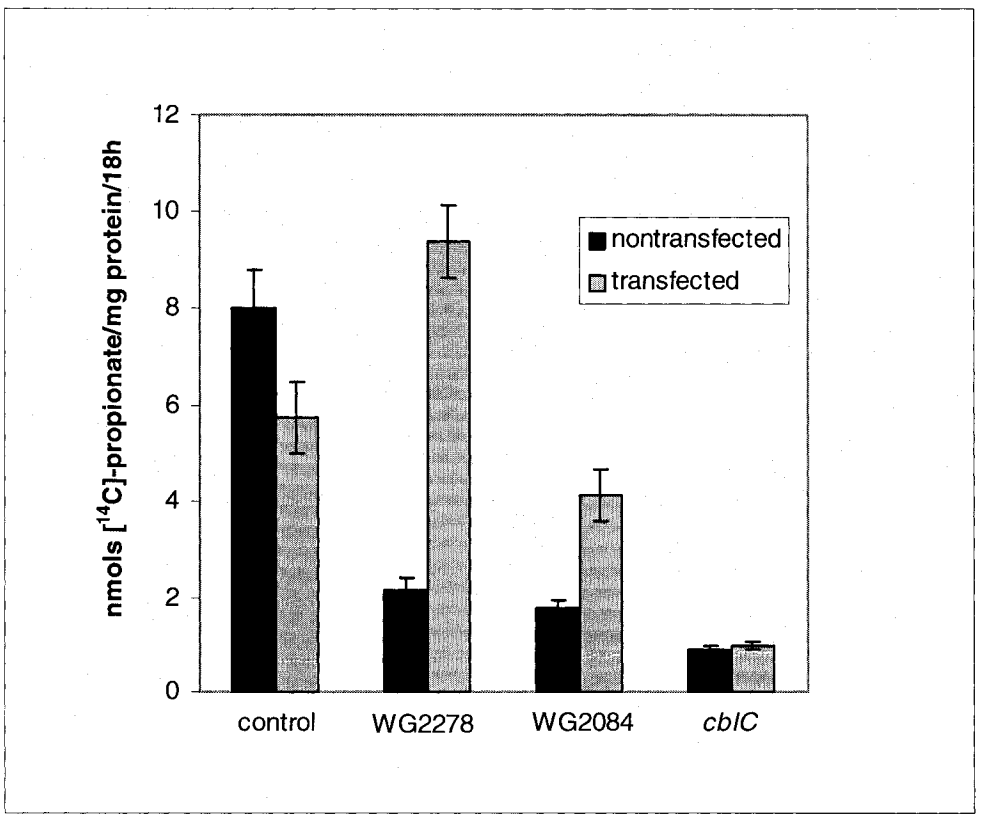
Figure 1 - Complementation analysis of *MCEE* deficient fibroblasts, both of which harbor the c.139C>T, p.R47X homozygous nonsense mutation in *MCEE* (WG2278 and WG2084). WG2278 and WG2084 were both fused with a *mut* cell line (WG3572) and with each other. [^{14}C]Propionate incorporation was measured in parallel in fused and unfused cultures. Increased [^{14}C]propionate incorporation observed in fusions with *mut* line but not between epimerase lines. Figure represents results of one experiment however the experiments were duplicated with similar results.



Transduction Experiments

Fibroblasts from WG2278, WG2084, a *cblC* cell line (WG3568), and a control cell line (MCH64) were infected with the *MCEE* construct. The [^{14}C]propionate incorporation for WG2278 and for WG2084 was significantly increased after infection compared to uninfected cells (p=0.0002 and 0.0041, respectively). In the *cblC* fibroblast cell line, infection with the *MCEE* construct had no effect on [^{14}C]propionate incorporation (p=0.429). The [^{14}C]propionate incorporation decreased slightly in control fibroblasts infected with *MCEE* (p=0.0425) (Figure 2).

Figure 2 - [^{14}C]Propionate incorporation in cultures of control cell line (MCH64), epimerase deficient WG2278 and WG2084, and a *cblC* cell line (WG3568) with and without infection of wild-type *MCEE* cDNA. Figure represents results of one experiment however the experiments were duplicated with similar results.



DISCUSSION

This project was undertaken to investigate the frequency of mutations in *MCEE* in patients who excreted moderate levels of methylmalonic acid, and in whom no cause had been identified. Interest in the role of mutations in the *MCEE* gene arose with the identification of a patient (WG2278) with methylmalonic aciduria of unknown etiology who was homozygous for a c.139C>T, p.R47X mutation in this gene (Dobson et al., 2006). In the present study, *MCEE* mutations were identified in five additional patients out of 229 studied. Two patients, both with decreased [^{14}C]propionate incorporation, were homozygous for the previously described c.139C>T, p.R47X mutation [one of these patients, WG2084, was simultaneously identified by Bikker et al., 2006]. Among the patients with normal [^{14}C]propionate incorporation, one was homozygous for a missense mutation c.178A>C, p.K60Q at a lysine residue that is conserved in mammals and nematodes (Kuhnl et al., 2005). Two other patients were heterozygous for a missense mutation affecting codon 143 of the *MCEE* gene c.427C>T, p.R143C. This represents a nonconservative change at an arginine that is conserved in animals, some archaea, and some bacteria (Kuhnl et al., 2005). One CEPH control was heterozygous for a sequence change in the *MCEE* gene that has not been previously described.

The reduced [^{14}C]propionate incorporation in two cell lines (WG2278 and WG2084) with the homozygous nonsense mutation was complemented by fusion with *cblA*, *cblB*, and *mut* fibroblasts but not by fusion with each other. Therefore, the decreased [^{14}C]propionate incorporation in these patients is likely the result of decreased epimerase function. This is further supported by the results of the infection studies; overexpression of wild-type epimerase in these cell lines corrected the cellular phenotype. An additional patient, homozygous for a missense mutation affecting a well-conserved amino acid (c.178A>C, p.K60Q), further supports defects in the epimerase as a cause of increased methylmalonic acid levels. The causal relationship between the heterozygous mutations in *MCEE* mutations and the methylmalonic aciduria seen in patients WG1476 and WG3270 has not been determined. It is not clear whether the heterozygous mutations in *MCEE* seen in patients WG1476 and WG3270 are the cause of their methylmalonic aciduria. It is noteworthy that there was a large discrepancy in methylmalonic acid values in these two patients. It is possible that heterozygosity for

both mutations in the *MCEE* gene and in other genes in the propionate pathway could contribute to the methylmalonic aciduria in these patients.

The results of the current study suggest that mutations in *MCEE* can be associated with increased excretion of methylmalonic acid, but are not a common cause. Including results presented in this current paper, four individuals (three families) have now been described that are homozygous for the same *MCEE* c.139C>T, p.R47X nonsense mutation (Dobson et al., 2006; Bikker et al., 2006); methylmalonic acid excretion has been detected in each of these individuals. However, it is not clear that the methylmalonic aciduria in these patients is consistently associated with clinical illness; if it is in fact associated there appears to be a large range in phenotype. The patient WG2278, reported by Dobson et al. (2006) had severe metabolic acidosis, but her sibling, as well as one of the patients reported here (WG2797) were clinically asymptomatic despite being homozygous for the same nonsense mutation. The serious clinical findings in patient WG2084 seem all to be associated with this patient's sepiapterin reductase deficiency (Bikker et al., 2006). The patient homozygous for the c.178A>C, p.K60Q missense mutation (WG2098) had a clinical picture that included of ataxia, deteriorated motor function, dysarthria, mild spastic paraparesis, and a reportedly normal MRI. The apparent lack of a distinct clinical presentation in patients with homozygous *MCEE* mutations is inconsistent with the strong conservation of the gene across large phylogenetic distance; one-sixth of the residues are identical in epimerases from eukaryotes, bacteria and archaea (Kuhnl et al., 2005), suggesting strong selection for the gene. The reason for this contradiction is not known.

Our findings contribute further to the debate over the requirement of the epimerase for cellular metabolism. We have shown that five patients with mild methylmalonic aciduria have mutations in the *MCEE* gene, and it seems likely that these mutations would be related to their methylmalonic acid excretion. As well, two cell lines with the same homozygous nonsense mutation in the epimerase did not complement each other. Furthermore, transduction of *MCEE* cDNA into two cell lines with the homozygous nonsense mutation resulted in correction of the cellular defect, indicating that mutations in the epimerase gene were causal of the observed phenotype. Overall, we conclude that a functional epimerase enzyme is required for normal methylmalonate

metabolism, and that a defect at this locus can result in, but is not a frequent cause of the excretion of increased amounts of methylmalonic acid.

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Watkins D: Vitamin B_{12} distributions and editing.

Rosenblatt DS: Principal Investigator.