

The Molecular Basis Of Glutamate Formiminotransferase Deficiency

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August, 2001

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment
of the requirements for the degree of

Masters of Science

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0-612-78893-8

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Abstract

Glutamate formiminotransferase deficiency (OMIM 229100) is an autosomal recessive disorder marked by clinical heterogeneity. The severe phenotype, first identified in patients of Japanese descent, includes high levels of formiminoglutamate (FIGLU) in the urine in response to histidine loading, megaloblastic anemia, and mental retardation. The mild phenotype is marked by high levels of FIGLU in the urine in the absence of histidine loading, mild developmental delay and no hematological abnormalities. The gene for human glutamate formiminotransferase-cyclodeaminase consists of 15 exons and is located at 21q22.3. The protein consists of a tetramer of dimers, with dimerization essential for both formiminotransferase and cyclodeaminase activity.

Genomic DNA extracted from cell lines from three patients with suspected glutamate formiminotransferase deficiency was analyzed by PCR and sequencing of individual exons. Cell lines WG 1758 and WG 1759 are from two siblings of Germanic descent. Both siblings are heterozygous for the mutations c457 C→T and c940 G→C. The c457 C→T changes a conserved arginine to a cysteine in a loop involved in the binding of formiminotetrahydrofolate to the enzyme. The c940 G→C mutation converts an arginine to a proline in an α -helix essential for the dimerization of the formiminotransferase domain. Cell line WG 1795 is from a patient of Danish descent. The patient appears to be hemizygous for a c1033 insG mutation. Quantitative PCR suggests the presence of a deletion on the other chromosome, which minimally encompasses exon 9. All of the FTCD gene changes were absent in 100 control individuals (200 alleles).

Sommaire

La déficience en glutamate formiminotransférase est une maladie récessive autosomale qui se manifeste par plusieurs différents états cliniques. Le phénotype sévère, identifié en premier lieu chez des patients d'origine japonaise, se caractérise par de hauts niveaux de formiminoglutamate (FIGLU) dans l'urine en réponse à l'accumulation d'histidine, par une anémie mégaloblastique et par un retard mental. Le phénotype atténué, quant à lui, se traduit par de hauts niveaux urinaires de FIGLU sans accumulation d'histidine, par une absence d'anormalités hématologiques, ainsi qu'un léger retard mental. Le gène de la protéine glutamate formiminotransférase-cyclodéaminase humaine est formé de 15 exons au locus 21q22.3. La protéine consiste en un tétramère de dimères. La dimérisation s'avère essentielle pour l'activité de la formiminotransférase et de la cyclodéaminase.

L'ADN génomique de trois lignées cellulaires provenant de 3 patients potentiellement atteints de déficience en glutamate formiminotransférase a été analysé par PCR et séquençage de chaque exon. Les lignées cellulaires WG 1758 et WG 1759 proviennent de 2 patients d'origine allemande ayant les mêmes parents. Ces 2 patients sont hétérozygotes pour les mutations c457 C→T et c940 G→C. La première change une arginine conservée pour une cystéine dans une boucle qui est impliquée dans la liaison du formiminotétrahydrofolate à l'enzyme. La deuxième mutation change une arginine pour une proline dans une hélice qui s'avère essentielle à la dimérisation du domaine formiminotransférase. La lignée cellulaire WG 1795 provient d'un patient d'origine danoise. Le patient semble être hémizygoté pour la mutation c1033 ins G. Des expériences de PCR quantitatif suggèrent la présence d'une délétion incluant au minimum l'exon 9 sur l'autre chromosome correspondant. Les mutations précédemment décrites n'étaient pas détectées chez 100 individus contrôles (200 allèles).

Rationale and Objectives of the Study

Glutamate formiminotransferase deficiency is an inborn error of folate metabolism which has been presumed to result from mutations in the gene for glutamate formiminotransferase-cyclodeaminase (FTCD). Although no mutations in FTCD have been identified to confirm or disprove this hypothesis, with the identification of the human FTCD, direct investigation of the gene sequences in affected individuals is possible.

The goal of this study was to identify the first known mutations responsible for glutamate formiminotransferase deficiency and to show that reduced function of glutamate formiminotransferase-cyclodeaminase is responsible for the disease. These goals were achieved by identifying and confirming mutations in three affected individuals. This study identified mutations by sequencing all 15 exons of FTCD from three patients with glutamate formiminotransferase deficiency. Each exon will be amplified by PCR and directly sequenced from genomic DNA isolated from 3 cell lines. Identified changes were confirmed as mutations by assessing their frequency in a panel of 100 normal individuals.

Glutamate formiminotransferase deficiency is a heterogeneous disease. Some patients present with a severe phenotype, which includes mental retardation, hematological abnormalities, elevated serum folate levels and urinary FIGLU excretion in the urine following histidine loading. Other patients present with a mild phenotype, which includes delayed speech development, hypotonia and urinary FIGLU excretion in the absence of histidine loading. Patients presenting with the mild phenotype do not have hematological abnormalities or elevated serum folate levels. Based on the urinary FIGLU levels, it was proposed that individuals with the mild form of the disease possess blocks in the formiminotransferase reaction, while

individuals with the severe form of the disease possess blocks in the cyclodeaminase reaction.

This study discusses whether the molecular basis of disease in the 3 patients fits this proposal.

Acknowledgements

I would first like to thank my supervisor, Dr. David S. Rosenblatt, for his efforts during my Masters Degree. Your guidance and criticism during the last two years has been most appreciated, and I hope to use what I learned from you in my future endeavours.

Another individual to whom I am in debt is Dr. David Watkins. Without your assistance in proofreading and criticizing my work, this thesis would have been much more difficult to assemble. In addition, I consider discussing science and ideas with you one of the best parts of the last two years.

I would like to thank Dr. Benjamin Raby for his invaluable assistance, ranging from technical issues to interpretation. Your resourcefulness and creativity helped make my research work much easier.

I would like to thank Dr. Brian Gilfix for his advice throughout the last two years, and for his suggestion of using quantitative PCR as an approach to one of research questions.

Last but not least, I would like to thank everyone with whom I have worked and interacted with during the last two years. All of you have offered me support and advice that has helped me immeasurably. In particular, I would like to thank Yasmin Karim, Angie Hosack, Nora Matiaszuk, Maria Galvez, Antonia Klintorinos, Xiao-ling Wang, and the staff of the Montreal Genome Centre.

List of Abbreviations

Dihydrofolate	DHF
Tetrahydrofolate	THF
5,10-methylenetetrahydrofolate	5,10-CH ₂ -THF
5,10-methenyltetrahydrofolate	5,10-CH ⁺ -THF
10-formyltetrahydrofolate	10-HCO-THF
5-formyltetrahydrofolate	5-HCO-THF
5-methyltetrahydrofolate	5-CH ₃ -THF
Nicotinamide adenine dinucleotide	NAD ⁺
Nicotinamide adenine dinucleotide phosphate	NADP ⁺
Intrinsic Factor	IF
Transcobalamin II	TCII
Formiminoglutamate	FIGLU
Uracil monophosphate	dUMP
deoxythymidine monophosphate	dTMP

Chapter #1
Overview of Folate Metabolism

Folates play a critical role in single carbon transfer reactions essential for the de novo synthesis of purines, pyrimidines and methionine and the degradation of histidine, serine and glycine. There are a number of confirmed inherited disorders of intracellular folate metabolism, including 5,10-methylenetetrahydrofolate reductase deficiency, methionine synthase deficiency (*cblG*), methionine synthase reductase deficiency (*cblE*), and glutamate formiminotransferase deficiency (Rosenblatt and Fenton, 2001). Folate deficiency is also one of the causes of megaloblastic anemia.

Megaloblastic anemia was first described in patients with pernicious anemia, which involves a block in the intestinal absorption of vitamin B₁₂. Anemias caused by vitamin B₁₂ deficiency are resolved by replacing the vitamin in the blood stream either through injections of liver extract or injections of the pure vitamin; however, Lucy Wills found forms of macrocytic anemia, a subset of megaloblastic anemia, which were not resolved by vitamin B₁₂ (Wills, 1931). It was eventually shown that the patients observed by Wills suffered from a dietary deficiency in folate. This was the first evidence that folate and vitamin B₁₂ metabolism were interconnected, an idea that was strengthened when pharmacological doses of folate were shown to alleviate some, but not all, of the clinical manifestations associated with vitamin B₁₂ deficiency (Hoffbrand and Weir, 2001; Cooper and Rosenblatt, 1995).

Chemical Structure of Folic Acid

Folic acid is the common name for pteroylglutamic acid, which consists of three major constituents, as shown in figure #1. The first constituent, 2-NH₂-4-OH-pteridine, is a bicyclic compound that can be substituted with various one carbon groups and can exist in different oxidation states. The second constituent, para-aminobenzoic acid, is found between the pteridine

and the glutamic acid moiety. The third constituent is glutamic acid, which is attached to para-aminobenzoic acid via an amide bond (Cossins, 1984).

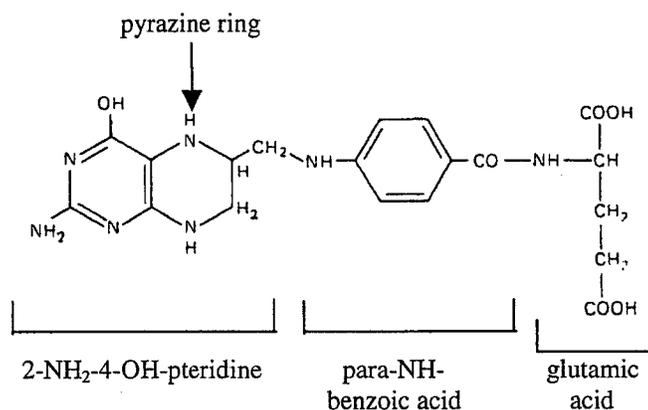


Figure #1: Chemical structure of folic acid. The left subgroup is 2-NH₂-4-OH-pteridine, the middle subgroup is para-aminobenzoic acid and the right subgroup is glutamic acid. Under physiological conditions, there is frequently more than one glutamic acid attached (Rosenblatt, 1995).

Folate within the cell exists conjugated to more than one glutamate residue forming a chain at one end of the molecule. Attached to each other via amide bonds at the γ -carbon, this polyglutamate chain varies in length depending on intracellular and physiological conditions. The terms pteroylpolyglutamate and folate polyglutamate refer to folates with more than one glutamate residue attached (Rosenblatt and Fenton, 2001).

Folate compounds can exist with the pyrazine ring of the pteridine moiety in three different oxidation states: a fully reduced form, 5,6,7,8-tetrahydrofolate; a partially oxidized form, 7,8-dihydrofolate; and a fully oxidized form, folic acid. Folic acid is not a naturally occurring form of the vitamin, and only is produced synthetically. 5,6,7,8-tetrahydrofolate is the only form that is biologically active, so organisms need enzymes to keep the molecule at its most reduced form (Rosenblatt and Fenton, 2001).

Folate carries the one carbon units between N^5 and N^{10} in three different oxidation states. The most oxidized one carbon unit is equivalent to formate, and is found in the forms N^5 -formylTHF, N^{10} -formylTHF, N^5 -formiminoTHF, and N^5,N^{10} -methenylTHF. The partially reduced single carbon unit is equivalent to formaldehyde, and is represented by N^5,N^{10} -methyleneTHF. The most reduced single carbon unit is equivalent to methanol, and is represented by N^5 -methylTHF (Voet and Voet, 1995).

Folate Translocation Into Mammalian Cells

Interest in folate translocation increased when it was demonstrated that there was linear relationship between the *in vitro* rate of uptake of methotrexate, a folate analog which acts as an inhibitor of dihydrofolate reductase, and the ability of the drug to kill cells *in vivo* (Kessel et al., 1965). Further research has demonstrated that two different transport systems exist within mammalian cells. The first, the reduced-folate carrier (RFC), mediates the uptake of reduced folates into cells. The second, the membrane-associated folate binding proteins (FR), is responsible for the uptake of oxidized folates.

The transport properties of the two systems have been well documented. The RFC system is selective for a reduced pyrazine ring, with substituted derivatives at N^5 of THF being the primary molecules targeted for transport. The system works best when its substrates are in the micromolar range. In contrast, the FR system is selective for oxidized pyrazine rings. Unlike the RFC, the FR system is capable of transporting folate when it is present in nanomolar concentrations. The two systems also differ in their transport kinetics, with the RFC having a 100 fold higher V_{max} compared to FR. Because of these observations, the RFC system has been referred to as the high capacity/low affinity transport system, while the FR system is called high affinity/low capacity transport system (Fan et al., 1992).

The two different systems were isolated from L1210 murine leukemia cells using biotin derivatives of methotrexate and folate. Amino acid comparisons indicate that the two proteins are similar, although the RFC receptor is a little more hydrophobic. Phospholipase C and peptide:N-glycosidase F treatment showed that FR proteins were heavily glycosylated at asparagine residues and are attached to phosphatidylinositol while the RFC transporters are embedded in the membrane and not glycosylated (Fan et al., 1992). Although the RFC transporters in L1210 murine leukemia cells were not found to be glycoproteins, this may not be true of all eukaryotic cell types. CEM-7A leukemia cells, a derivative of CCRF-CEM leukemia cells which express up to 30 times more RFC protein, were found to contain glycosylated RFC protein (Freisheim et al., 1992).

The discovery of RFC encoding cDNAs in lower organisms provided the foundation for cloning the human version of the gene. Homology comparisons allowed the isolation of a human cDNA clone, which could restore methotrexate uptake in a Chinese hamster cell line deficient in this function. The cDNA clone was 2863 bp in length with an open reading frame of 1770, and it was predicted that it encoded for either the RFC protein or an auxiliary protein which interacted with the transporter (Williams and Flintoff, 1995). Parallel research efforts on the same gene showed that the murine form of RFC could be used to restore RFC function in human breast cancer cells. Using a human testis cDNA library, the human homolog of the gene was mapped to 21q22.2-q22.3. This gene has been named RFC-1 (Moscow et al., 1995; Wong et al., 1995).

Expression studies indicated that some methotrexate resistant cell lines showed a decrease in RFC-1 mRNA expression when compared to their methotrexate sensitive parental lines. When it was shown that RFC-1 expression could restore methotrexate sensitivity, the

RFC-1 gene was recognized as one of the causes of methotrexate resistance (Moscow et al., 1995; Wong et al., 1995).

An interesting feature of rodent and human RFC-1 transcripts is their heterogeneity. Several RFC transcripts are produced from a single locus and are a result of multiple transcriptional start sites and variable splicing of two alternative non-coding exons; which of the two exons is included depends on the transcriptional start site. The different transcripts do not affect the structure of the RFC protein, so it is possible that the alternative 5'-UTRs may affect post-transcriptional regulation in some way (Zhang et al., 1998).

Knockout mice for RFC-1 have been created. Without intervention, RFC-1 embryos died at embryonic day 9.5. This can be somewhat overcome by supplementing the mother subcutaneously with 1 mg of folic acid per day. With supplementation, only 10% of all embryos develop to term, and these mice lack erythropoiesis in bone marrow and spleen. In addition, the mice suffer from lymphoid depletion. These observations suggest that non-functional RFC-1 in developing animals results in hematopoietic failure (Zhao et al., 2001).

Two common human FR cDNA isoforms, FR- α and FR- β , have been cloned. Both of these genes are found in a cluster on chromosome 11q13.2-q13.5. Both genes have similar structures, but different tissue-specific transcriptional regulatory elements and 5'-UTRs (Lacey et al., 1989; Elwood, 1989; Elwood et al., 1997). Initial experiments linked folate uptake by the FR proteins to potocytosis, a process where there is receptor-mediated endocytosis of molecules in the absence of clathrin-coated vesicles. This is different from other receptor-mediated ingested compounds, where the endocytosed vesicle is dependent on clathrin for its formation. The proposed mechanism for potocytosis is as follows: folates bind to the phosphatidylinositol-anchored receptor, which is then internalized by potocytosis. This was followed by acid-induced

release of the folate molecule, and the recycling of the receptor to the surface. A concerted model between the two receptors has also been proposed (Antony, 1996).

Folic acid supplementation before pregnancy is known to reduce the risk of birth defects, although the reason why is still unknown (MRC, 1991). Knockout mice for *Folbp1*, the murine analog of FR- α , have severe morphologic abnormalities and fail to develop past day 10 in utero. Supplementing the mothers with 5-formyltetrahydrofolate (folinic acid) during the full term of pregnancy prevented this phenotype. These results suggest that defects in folate transport between the mother and developing fetus may be one of the causes of developmental defects (Piedrahita et al., 1999).

Synthesis and Degradation of Pteroylpolyglutamates

Folate-dependent enzymes within cells require a critical concentration of intracellular folate for function. The amount of folate required to sustain optimal growth of cell cultures depends on cell type. Human fibroblasts require 50 nM of intracellular tetrahydrofolate (Hilton et al., 1979) while human leukemia cell line K562 requires about 1 μ M of non-methylated tetrahydrofolate for optimal growth (Watkins and Cooper, 1983). These concentrations are far below the K_m determined for the folate-dependent enzymes using folate monoglutamate substrates. Increase in polyglutamate chain length, however, decreases their apparent K_m . Therefore, extending the polyglutamate chain is the way a cell overcomes its need for higher than physiological concentrations of folate. The optimal chain length varies for each folate-dependent enzyme (Rosenblatt and Fenton, 2001).

Since the chain length of pteroylpolyglutamates almost always exceeds the size limit that the folate transport system can handle, cells must have evolved a mechanism to modify folates to their physiologically active polyglutamate forms. The enzyme that catalyzes this reaction is

pteroylpolyglutamate synthetase (FPGS), which uses the energy of ATP hydrolysis to add L-glutamate molecules one at a time to tetrahydrofolate derivatives. The enzyme forms a peptide bond between the γ -carboxyl of the already attached glutamate and the amino group of the new glutamate. In mammals, there is both a cytoplasmic and mitochondrial version of the enzyme (McGuire and Coward, 1984).

The mammalian synthetase appears to have three general properties. The first is that substrates for the enzyme must be either tetrahydrofolate or formyltetrahydrofolate; 5-methyltetrahydrofolate is not a substrate for the synthetase (Shane, 1989). The second is that the final distribution of folylpolyglutamates in the cell is dependent on the differing abilities of each possible form to act as a substrate for the synthetase (Foo and Shane, 1982). The third property is that activity decreases as the chain length increases; this sets an upper limit to the length of polyglutamate chains within mammalian cells (Foo and Shane, 1982). Glutamate chain length within the cell is also dependent on the extracellular concentration of folate. At low extracellular concentrations, the cell synthesizes longer polyglutamate chains on its folate molecules and at high extracellular concentrations, the synthesized chains are much shorter (Brody et al., 1979).

There are specific cell lines, which have deficient pteroylpolyglutamate synthetase function. A mutant Chinese hamster ovary cell line, AUXB1, accumulates normal levels of intracellular monoglutamate folates but less than 1.5% of the expected levels of polyglutamate folates. The cell line is auxotrophic for glycine, adenine, thymidine and methionine, all of which are products of folate metabolism. The absence of pteroylpolyglutamates is believed to be the cause of the phenotype (McBurney and Whitmore, 1974). Although there is a methotrexate-resistant human breast carcinoma cell line that is defective in the synthesis of methotrexate

polyglutamate, to date there are no humans diagnosed with pteroylpolyglutamate synthetase deficiency (Rosenblatt and Fenton, 2001).

Cloning of the cDNA for human FPGS was performed by expressing human cDNAs in *folC*, an *E. coli* mutant that is unable to synthesize pteroylpolyglutamates. The complementing *folC* cDNAs were also shown to complement AUXB1 Chinese hamster ovary cells (Garrow et al., 1992). The genomic structure of FPGS has been elucidated. A single gene at chromosome 9cen-q34 encodes both the cytoplasmic and mitochondrial forms of the enzyme. The forms of the protein differ in their transcriptional start sites, which leads to different N-terminal signal sequences in the protein. Expression studies have indicated that the gene is regulated by both the type of tissue and by the level of cellular proliferation (Taylor et al., 1995).

The polyglutamate chains of pteroylpolyglutamates require special enzymes for their degradation as standard proteolytic enzymes do not recognize the γ -peptide bond. The enzymes, which target pteroylpolyglutamates, are known as pteroylpolyglutamate hydrolases or conjugases. There are two distinct forms within mammals. The first one is located within the intestinal brush border and plays an essential role in folate uptake as pteroylpolyglutamates must be hydrolyzed to monoglutamate or diglutamate forms if they are to cross the intestinal mucosa (Rosenblatt and Fenton, 2001). The second form is intracellular and differential centrifugation indicates that it is lysosomal (Silink and Rowe, 1975). Since the intracellular enzyme is non-specific as to its targets, it may be possible to regulate intracellular levels of the various folate derivatives by controlling which type enters the lysosomes for glutamate chain degradation (Rosenblatt and Fenton, 2001).

Both the rat and human γ -glutamyl hydrolase have been cloned. Using rat H35 hepatoma cells that secreted γ -glutamyl hydrolase, it was possible to purify the rat version of the protein

and identify its cDNA (Yao et al., 1996). The rat cDNA then provided the necessary criteria to search a human EST database. It was determined that the human γ -glutamyl-hydrolase has 67% amino acid similarity when compared to the rat and that the first 24 amino acids preceding the N-terminus of the protein constitute a structural motif similar to those seen in signal sequences (Yao et al., 1996).

Metabolic Pathways of Folate Metabolism

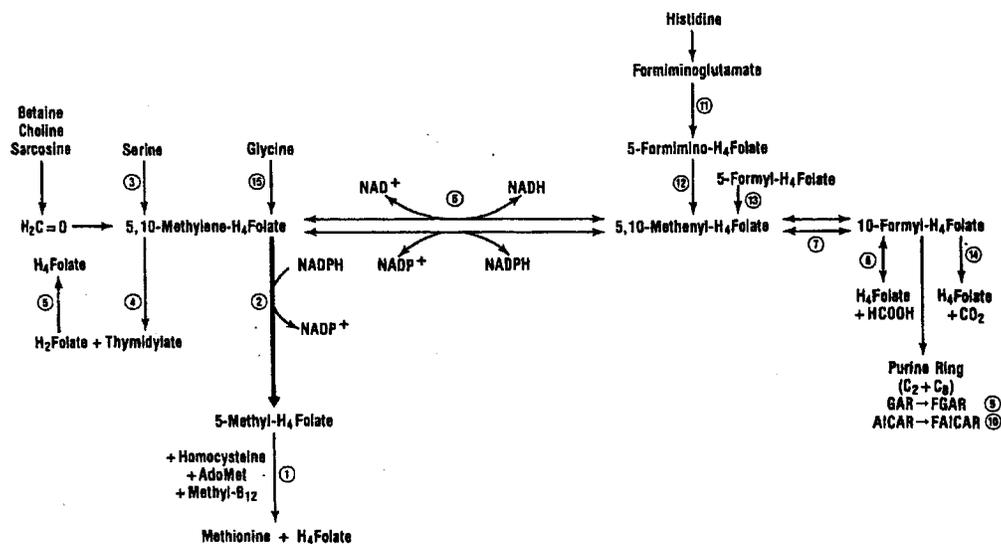


Figure #2: Metabolic Pathways of Folate Metabolism. 1. Methionine synthase; 2. 5,10-methylenetetrahydrofolate reductase; 3. Serine hydroxymethyl transferase; 4. Thymidylate synthase; 5. Dihydrofolate reductase; 6. Methylenetetrahydrofolate dehydrogenase; 7. Methenyltetrahydrofolate cyclohydrolase; 8. 10-Formyltetrahydrofolate synthase; 9. GAR transformylase; 10. AICAR transformylase; 11/12. Glutamate formiminotransferase-cyclodeaminase; 13. 5,10-methenyltetrahydrofolate synthetase; 14. 10-formyltetrahydrofolate dehydrogenase; 15. Glycine cleavage system (Rosenblatt and Fenton, 2001)

Intracellular folate metabolism can be broken down into three distinct stages. The first stage involves reduction of the pteridine ring and the addition of one-carbon moieties. The second stage involves the interconversion of one-carbon substituted folate molecules. The third stage is the utilization of the one-carbon substituted folates for purine synthesis, pyrimidine synthesis and methionine synthesis.

Reduction of Folate Molecules

Dihydrofolate reductase (DHFR) catalyzes the reduction of 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate using NADPH as the source of reducing electrons. The enzyme also reduces folic acid to 5,6,7,8-tetrahydrofolate using two molecules of NADPH, but this occurs at a much slower rate. This enzyme is present in all dividing cells because of its two essential roles in folate metabolism. First, DHFR ensures that all folate is reduced to its active form. Second, thymidylate synthesis results in the oxidation of 5,10-methylenetetrahydrofolate to dihydrofolate. Dividing cells, therefore, require a functionally active DHFR to prevent thymidylate synthesis from trapping the cell's folate pool in an unusable form. Anti-tumor drugs, such as aminopterin and methotrexate, inhibit DHFR activity. This prevents the cell from synthesizing the necessary bases for DNA replication, killing dividing cells (Blakely, 1984).

Protein structure has been investigated in DHFR from a variety of organisms. There are a number of invariant residues, and homology appears to be the highest in the N-terminal end (Blakely, 1984). Although DHFR is structurally similar in all organisms, subtle differences between organisms lead to significant differences in affinity for various substrates and inhibitors. For example, the inhibitor trimethoprim has a higher affinity for the bacterial forms of DHFR because it is capable of forming favorable interactions with the side chains surrounding the active site. The vertebrate version of DHFR has the side chains oriented differently, raising the K_m for trimethoprim substantially (Matthews et al., 1985). The reverse is true for phenyltriazine derivatives, which selectively inhibit vertebrate DHFRs. In this case, the side chains surrounding the active site of vertebrate forms are ideally situated for favourable interactions where similar interactions are not possible in bacterial versions (Volz et al., 1982).

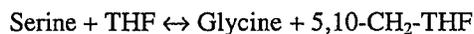
Human DHFR was mapped to human chromosome 5q11-q22 using somatic cell complementation with the DHFR mutant Chinese hamster cell line DXB11 (Funanage et al., 1984). In addition to the functional version on chromosome 5, there are also four intronless pseudogenes on different chromosomes. The h-DHFR- ψ_1 is identical to h-DHFR and is present in its entirety in some individuals or completely absent in others (Anagnou et al., 1984). Allelic frequency for h-DHFR- ψ_1 is 94% in Mediterraneans, 77% in Asian Indians, 67% in Chinese, 57% in Southeast Asians, and 32% in African Americans. This suggests that transposition of the pseudogene occurred before the inception of human racial groups (Anagnou et al., 1988).

It has been observed that if mammalian cells are exposed to progressively increasing concentrations of methotrexate, a potent inhibitor of DHFR, the cells may show a selective amplification of DNA sequences encoding for the DHFR gene. This selective amplification is known to occur in two forms, with only one of them associated with a stable increase in the number of DHFR sequences. In cells where the amplified gene number remains stable following the removal of methotrexate, it was determined that the amplified genes were located within the chromosomes of the cell. In cells where the amplified gene number changed following the removal of methotrexate, the duplications were associated with small paired chromosomal elements called "double minute" chromosomes. Changes in DHFR gene number in these cells are due to unequal segregation during mitosis. Without centromeres, these double minute chromosomes are unable to segregate properly, leading the changes in gene number every generation (Kaufman et al., 1979).

Sources of One-Carbon Units for Folate Metabolism

Degradation of serine, glycine and histidine provide the one-carbon units for folate metabolism, with serine and glycine being the greatest contributors. Serine is degraded by cytoplasmic and a mitochondrial form of serine hydroxymethyltransferase while glycine is degraded by the glycine cleavage system located in the mitochondria. Breakdown of histidine produces formiminoglutamate, which is degraded by glutamate formiminotransferase-cyclodeaminase. Glutamate formiminotransferase-cyclodeaminase is the subject of chapter 3 of this thesis.

Serine hydroxymethyltransferase (SHMT), a periodical-phosphate dependent enzyme, catalyzes the breakdown of L-serine to glycine via the following reaction:



This enzyme is present in both the cytoplasm and mitochondria. Purified forms have molecular weights of 220,000 DA and consist of four identical subunits. In mammalian cells, both 5-methyltetrahydrofolate and 5-formyltetrahydrofolate serve as feedback inhibitors of SHMT (Schirch, 1984). It has been reported that a Chinese hamster ovary cell line deficient in the mitochondrial SHMT, known as the *GlyA* complementation group, is auxotrophic for glycine (Chasin et al., 1974). This suggests that the mitochondrial SHMT plays an important role in maintaining glycine levels within the cell.

Human cDNAs for cytosolic and mitochondrial SMHTs have been identified by expressing human cDNA libraries in *E. coli GlyA* mutants. The gene encoding the cytosolic version has been localized to 17p11.2 while the mitochondrial version is located at 12q13. Comparison of the two genes reveals a high degree of nucleotide sequence identity. Both genes

are surrounded by keratin encoding genes, suggesting that the two forms of SHMT arose from a duplication event (Garrow et al., 1993). The genomic structure and regulation of the mitochondrial SHMT have been studied. The gene is 4.5 kB and contains 10 introns and 11 exons. The promoter regions lack TATA consensus sequences but contain binding sites for Sp-1 and AP-2. Transcription of the gene can begin at multiple sites, with the signal sequence coming from one of two alternatively spliced exons. It was shown in culture that mRNA levels are not affected by decreases in glycine, serine, folate, thymidylate or purines, suggesting that these metabolites do not regulate transcription (Stover et al., 1997).

In mitochondria, glycine can be broken down to CO_2 and ammonia by the glycine cleavage system, producing 5,10-methylenetetrahydrofolate in the process. The glycine cleavage system involves four protein components that have been referred to as P-protein, H-protein, L-protein and T-protein. The function of these four proteins has been described (Hiraga and Kikuchi, 1980). The first step is catalyzed by the pyridoxal-phosphate dependent P-protein, which decarboxylates glycine to CO_2 and methylamine. The methylamine is then transferred to a lipic acid group on the H-protein. During the transfer, the one-carbon from methylamine is oxidized to formyl equivalency. The T-protein then transfers the formyl group to tetrahydrofolate to form 5,10-methylenetetrahydrofolate. The final step is the reoxidation of the reduced lipoate on the H-protein by the L-protein and NAD (Schirch, 1984).

Glycine, methionine and other nitrogen sources through a control element containing a core CTTCTT motif control expression of the genes responsible for glycine degradation in *Saccharomyces cerevisiae*. Since the presence of folate is essential for transcription, it has been postulated that the binding of transcription factors to this motif is modulated by 5-methyltetrahydrofolate levels (Hong et al., 1999; Piper et al., 2000). Further experimental work

in yeast has indicated that the genes involved in glycine degradation are co-regulated with the cytoplasmic serine hydroxymethyltransferase. The cytoplasmic SHMT is the primary provider of one-carbon units for 5,10-methylenetetrahydrofolate in the cell, and the glycine cleavage system interacts with SHMT to maintain the appropriate levels of 5,10-methylenetetrahydrofolate. In times of depleted 5,10-methylenetetrahydrofolate, cells upregulate the genes involved in the glycine cleavage system, compensating for shortage of one-carbon units derived via cytosolic SHMT. During times of glycine surplus, the glycine cleavage system genes are also upregulated in order to spare the breakdown of serine to glycine and to provide the necessary one-carbon units for folate metabolism. The presence of excess glycine acts as an allosteric inhibitor of the cytoplasmic SHMT (Piper et al., 2000).

There is also experimental evidence that not all one-carbon units are equivalent in yeast. One-carbon units generated by the cytoplasmic SHMT are mainly directed toward methionine biosynthesis, while one-carbon units generated by the glycine cleavage system are mainly directed toward purine biosynthesis (Piper et al., 2000).

Interconversion of Substituted Tetrahydrofolates

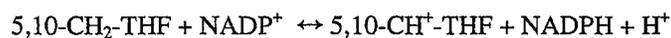
Substituted tetrahydrofolates can be converted into a number of folate derivatives in order to meet the demands of intracellular metabolism. In particular, cells require 5,10-methylenetetrahydrofolate, 10-formyltetrahydrofolate and 5-methyltetrahydrofolate for thymidylate, purine and methionine biosynthesis respectively. The folate interconversion pathway ensures that the cell can generate the required amounts of the above compounds.

Two different multifunctional enzymes systems carry out the interconversion of substituted tetrahydrofolates. A trifunctional polypeptide possesses 5,10-methylenetetrahydrofolate dehydrogenase, 5,10-methenyltetrahydrofolate cyclohydrolase, and

10-formyltetrahydrofolate synthetase which carry out reactions 6, 7 and 8 in figure #2 (Tan et al., 1977). This enzyme is found only within the cytosol (Mejia and Mackenzie, 1988), and also permits the release and capture of formate (Mackenzie and Tan, 1980) in addition to interconversion of 5,10-methylenetetrahydrofolate and 10-formyltetrahydrofolate. Somatic cell hybridization and in situ hybridization was used to localize the human trifunctional enzyme to chromosome 14q24, and identify a pseudogene at Xp11 (Rozen et al., 1989).

There is also a bifunctional enzyme which exists in mammals, however, it is only detected in undifferentiated cells or transformed cells. This enzyme encodes a protein with a 5,10-methenyltetrahydrofolate cyclohydrolase, and a 5,10-methylenetetrahydrofolate dehydrogenase (Mejia and Mackenzie, 1985).

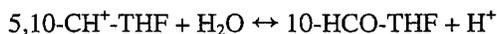
5,10-methylenetetrahydrofolate dehydrogenase catalyzes the following reversible reaction:



The enzyme shows Michaelis-Menten properties and the K_m values are very similar when comparing enzymes from different organisms (Mackenzie, 1984). This reaction catalyzes the first step in the generation of 10-formyltetrahydrofolate, a key molecule in de novo purine synthesis, from 5,10-methylenetetrahydrofolate. The enzyme does not release 5,10-methenyltetrahydrofolate but instead channels it to the cyclohydrolase, which catalyzes the next step (Drummond et al., 1983). The enzyme is allosterically inhibited by 10-formyltetrahydrofolate (Dev and Harvey, 1978). It has also been reported that the enzyme is inhibited by purine nucleotides. 5,10-methylenetetrahydrofolate dehydrogenases isolated from *S. typhimurium* are strongly inhibited in the presence of ATP, GTP, GDP and ITP, all of which are final or

intermediate products in de novo purine biosynthesis (Dalal and Gots, 1967). These inhibition properties are not present in all species.

5,10-methenyltetrahydrofolate cyclohydrolase catalyzes the following reversible reaction:



In vitro, the reaction is highly pH dependent, with products favoured in neutral or basic solutions and the reactants favoured in acidic situations. In vitro assays indicate K_m values much higher than physiological concentrations. This may be the reason why a multifunctional enzyme with substrate channeling has evolved (Mackenzie, 1984).

10-formyltetrahydrofolate synthetase extracts toxic formate from the cell and attaches it to tetrahydrofolate via the following reaction:



10-formyltetrahydrofolate synthetase requires monovalent cations for maximal activity. These cations are believed to affect the quaternary structure of the enzyme and affect the K_m values (Himes and Wilder, 1965). The equilibrium values favour the formation of 10-formyltetrahydrofolate ($K_{eq}=40$), which allows mammalian cells to effectively detoxify formate released from other metabolic pathways (Mackenzie, 1984).

The trifunctional peptide has been crystallized, helping elucidate the channeling and binding properties of the enzyme (Allaire et al., 1996). Residues arginine-173 and serine-197 are within 3Å of bound NADP and site-specific mutagenesis indicates that both residues affect the

K_m of the substrate (Pawelek et al., 2000). The dehydrogenase and cyclohydrolase share a single folate-binding site (Smith and Mackenzie, 1985). The catalytic residues have been determined. Lysine-56 and tyrosine-52 are the important residues for the dehydrogenase reaction, with lysine-56 is involved in the catalysis and tyrosine-52 involved in the orientation of 5,10-methylenetetrahydrofolate. For the cyclohydrolase reaction, both serine-49 and cysteine-147 are believed to chemically activate water, a process necessary for reaction completion (Schmidt et al., 2000).

The bifunctional enzyme has only been detected in embryonic, undifferentiated and transformed cells (Mejia and Mackenzie, 1985). The enzymatic properties were determined by purifying the protein 6000 fold from Ehrlich ascites cells. SDS-polyacrylamide electrophoresis in the presence of cross-linking reagents indicates that the bifunctional enzyme exists as a dimer within the cell (Mejia et al., 1986). Subcellular fractionation and Western analysis of murine cells show that the bifunctional enzyme is located in the mitochondria, while the trifunctional enzyme is located in the cytosol (Mejia and Mackenzie, 1988).

The dehydrogenase of the bifunctional enzyme catalyzes the same reaction as the one on the trifunctional enzyme, except it uses NAD^+ as the electron acceptor instead of $NADP^+$. In addition to being NAD^+ specific, the dehydrogenase requires a divalent cation for activity and is stimulated in the presence of organic phosphate. Studies of substrate affinity indicate that the dehydrogenase shows a greater affinity for folates with higher polyglutamate chains (Mejia et al., 1986). Based on in which cells the enzyme was expressed, it was proposed that the NAD^+ dependent methylenetetrahydrofolate dehydrogenase's primary role was to promote purine biosynthesis. It was also suggested that the presence of the bifunctional enzyme would greatly reduce the amount of 5-methyltetrahydrofolate within the cell, thus contributing to the

methionine-dependence phenotype observed in many tumor lines (Mejia and Mackenzie, 1985). The cyclohydrolase activity of the bifunctional enzyme shows the same characteristics as its counterpart in the trifunctional peptide.

There is also three other important interconversion reactions which are not part of the trifunctional enzyme. The first, 5,10-methylenetetrahydrofolate reductase catalyzes the following reaction within the cell:



A homogenous preparation of methylenetetrahydrofolate reductase from pig liver has been characterized. Each monomer is a flavoprotein containing one non-covalently linked molecule of FAD, with the active enzyme being a dimer of identical subunits. It has been determined that each subunit of the methylenetetrahydrofolate reductase has a molecular weight of 77 kDa. The physiological reaction is essentially irreversible, with the overall equilibrium ratio being 10^7 in favour of the products (Daubner and Matthews, 1982). The human methylenetetrahydrofolate reductase has been cloned and is located at 1p36.3 (Goyette et al., 1994).

Methylenetetrahydrofolate reductase has two spatially distinct subunit domains. Cleavage with 1% trypsin results in two fragments of 39 and 36 kDa. When the N-terminal fragment was studied, it was found to contain catalytic activity even in the presence of the enzyme's inhibitor, S-adenosylmethionine. This observation lead to the conclusion that the N-terminal domain of the protein is catalytic, while the C-terminal domain is regulatory. Scanning transmission electron microscopy indicates that the protein does not alter its overall structure in the presence of its inhibitor. (Matthews, 1984).

The active-methyl cycle is shown in figure #3, and plays an important role in the regulation of methylenetetrahydrofolate reductase.

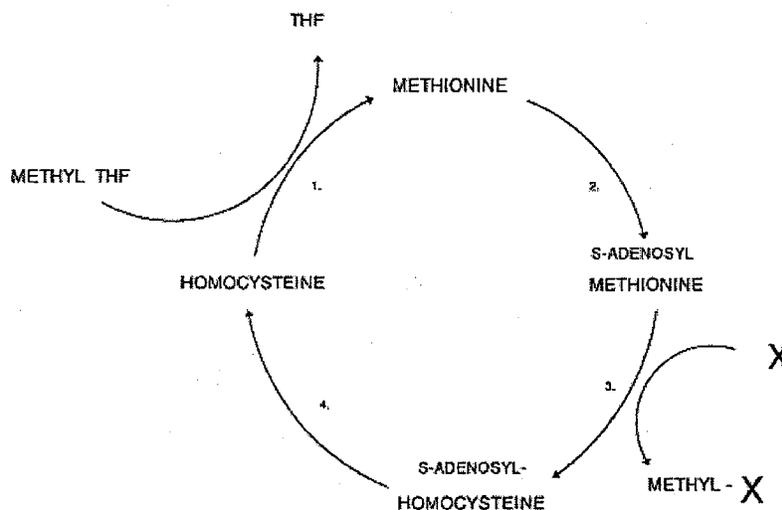


Fig #3: Active methyl cycle. 1) Homocysteine is converted to methionine by methionine synthase by using 5-methyltetrahydrofolate as a methyl source. 2) Methionine is converted to S-adenosylmethionine by methionine adenosyltransferase. This gives the sulfur atom a positive charge, allowing the methyl substituent to become a good leaving group. 3) The methyl group is transferred to a variety of compounds by methyltransferases. Two examples are acetylserotonin methyltransferase and nicotinate methyltransferase. 4) S-adenosylhomocysteine is converted to homocysteine by adenosyl homocysteinase (Watkins, unpublished).

Although binding of S-adenosylmethionine inhibits the enzyme, it has been shown that S-adenosylhomocysteine competes for the same site. Unlike S-adenosylmethionine, S-adenosylhomocysteine does not inhibit the enzyme. As a result, mammalian methylenetetrahydrofolate reductase is actually regulated by the ratio of the two compounds. If there are low levels of homocysteine, then methionine levels are high and it is not necessary to produce more 5-methyltetrahydrofolate. High levels of methionine are reflected by a high S-adenosylmethionine/S-adenosylhomocysteine ratio, which inactivates the reductase. If homocysteine levels are high, then the S-adenosylmethionine/S-adenosylhomocysteine ratio will be low, activating the reductase (Kutzbach and Stokstad, 1971).

The second interconversion reaction not on the trifunctional peptide is catalyzed by 10-formyltetrahydrofolate dehydrogenase, which oxidizes the single carbon unit attached to 10-formyltetrahydrofolate, allowing it to be released as carbon dioxide. This enzyme is present only in the liver and it is postulated to that its role is to maintain sufficient levels of unsubstituted tetrahydrofolates so there can be continued acceptance of single carbon units in other folate dependent reactions.

The third interconversion reaction not catalyzed by the trifunctional peptide is 5,10-methenyltetrahydrofolate synthetase, which uses ATP to convert 5-formyltetrahydrofolate (folinic acid) to 5,10-methenyltetrahydrofolate. Folinic acid is sometimes used as a treatment against methotrexate toxicity as it allows cells to meet their folate requirements when DHFR is inhibited (Rosenblatt and Fenton, 2001).

Much of the information about the enzymatic properties of 5,10-methenyltetrahydrofolate synthetase comes from purified enzyme from rabbit liver. The enzyme exists as a monomer with a molecular weight of 28,000 and is sensitive to pH and ionic strength. Initial velocity studies suggest that the enzyme mechanism is sequential and that the substrates bind randomly. These enzymatic experiments have contributed to the theory that the enzyme serves as a salvage pathway to generate metabolically active one-carbon units from 5-formyltetrahydrofolate (Hopkins and Schirch, 1984). In the cell, 5-formyltetrahydrofolate can be generated by the hydrolysis of 5,10-methylenetetrahydrofolate to 5-formyltetrahydrofolate by action of the serine hydroxymethyltransferase (Stover and Schirch, 1990)

The human 5,10-methenyltetrahydrofolate synthetase has been cloned by purifying the protein and using conserved amino acid sequences to generate degenerate primers for reverse transcription and PCR from mRNA extracted from human liver. The human 5,10-

methenyltetrahydrofolate-synthetase cDNA is 872 bp in length, encoding for 203 amino acids. There is a consensus ATP binding site from amino acids 147-151, and there is a potential Mg^{+2} binding site at amino acids 135-140 (Dayan et al., 1995).

Purine And Pyrimidine Synthesis

Folate metabolism plays a critical role in the de novo synthesis of purines and pyrimidines as it provides essential one-carbon units to both pathways. 10-formyltetrahydrofolate contributes a formyl group twice to the growing purine ring, while 5,10-methylenetetrahydrofolate contributes a single carbon unit to dUMP to form thymidine. Synthesis of purines and pyrimidines is an essential part of cellular growth, making the pathways ideal targets for anti-cancer therapy.

The origins of the individual atoms in a purine ring have been clearly defined and appear to be conserved throughout evolution. Glycine contributes carbons 4 and 5 as well as nitrogen 7. Carbon 6 is derived from carbon dioxide, which becomes carbonate within the cell. The amide substituent of glutamine provides nitrogens 3 and 9, while the amine of aspartate provides nitrogen 1. Carbons 2 and 8 are provided by 10-formyltetrahydrofolate in reactions catalyzed by two different enzymes (Voet and Voet, 1995).

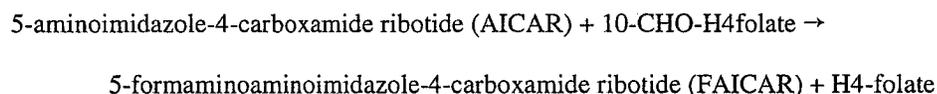
10-formyltetrahydrofolate is first used in step 4 in purine synthesis via the following reaction catalyzed by GAR transformylase:



X-ray crystallography of GAR transformylase indicates that the GAR amine is in close proximity to the N^{10} atom of THF. This is further supported by enzymatic studies, which suggest that the GAR transformylase reaction involves a nucleophilic attack of the GAR amine, by the formyl

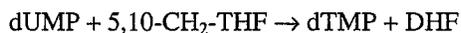
group of THF (Almassey et al., 1992). GAR transformylase is part of a trifunctional peptide encoded by a gene located on chromosome 21. Interestingly, one of the other functions of the trifunctional peptide is AIR synthetase, another step in purine biosynthesis (Hards et al., 1986).

10-formyltetrahydrofolate is used again in step 10 in the following reaction catalyzed by AICAR transformylase:



The compound 5-deazatetrahydrofolate can inhibit both GAR transformylase and AICAR transformylase. (Voet and Voet, 1995). AICAR transformylase is part of a bifunctional enzyme, which also encodes for IMP cyclohydrolase, the final step in purine biosynthesis. The gene has been cloned and is located on chromosome 2 (Rayl et al., 1996).

Thymidine is synthesized from deoxyuridine by thymidylate synthase using 5,10-methylenetetrahydrofolate as a methyl donor in the following reaction:



The transferred methylene group from 5,10-methylenetetrahydrofolate is reduced to a methyl group at the expense of oxidizing tetrahydrofolate to dihydrofolate. Because of its critical role in DNA synthesis thymidylate synthase is a target for a number of different antitumor agents. 5-fluorouracil and 5-fluoro-2'-deoxyuridine are two examples (Voet and Voet, 1995; Hardy et al., 1987). Somatic cell hybridization between a human lymphoblast cell line and Chinese Hamster Ovary cell line deficient in thymidylate synthase activity showed that the human version of the

gene was on chromosome 18 (Ledbetter et al., 1984). This allowed for the identification of genomic phage clones, which contained the gene (Takeishi et al., 1989).

Methionine Biosynthesis

Folate metabolism is tightly linked to intracellular methionine biosynthesis because it is the supplier of the methyl group that will be added onto homocysteine to generate methionine. Methionine biosynthesis requires three enzymes in two different steps. The first is the generation of 5-methyltetrahydrofolate by 5,10-methylenetetrahydrofolate reductase. The second is the addition of the methyl group to homocysteine, which is catalyzed by methionine synthase. Methionine synthase uses methylcobalamin as a cofactor. In the process of methylating homocysteine, the cobalamin cofactor can be oxidized from +1 to +2, resulting in the inactivation of the cofactor. Oxidized cobalamin is reduced back to +1 by methionine synthase reductase.

The main physiological function of methionine synthase (MS) is to remethylate homocysteine to produce methionine. This allows the cell to generate a constant supply of S-adenosylmethionine, an important methyl donor within the cell. The reaction also prevents the accumulation of potentially toxic homocysteine and S-adenosylhomocysteine. Cloning of the human gene was done in 1996 simultaneously by three different research groups. All groups relied heavily on sequences obtained in lower organisms. The gene is found on chromosome band 1q43, near the telomeric region, with a cDNA length of 7.2 kB.

The first group used specific regions of homology which were conserved in known MS genes cloned from different organisms and the cobalamin binding site of the human methylmalonyl-CoA mutase to design primers which could be used for RT-PCR, inverse PCR and sequencing. By assembling the experimental sequences in order based on primer location,

the complete cDNA sequence was derived (Leclerc et al., 1996). The second group took a very similar approach, but used the PCR product derived from the conserved primers as a probe in a human hepatoma cDNA library to retrieve a full length MS cDNA (Li et al., 1996). The third group took the same approach as the second except that a HepG2 cDNA library was scanned (Chen et al., 1996).

Studies of the bacterial enzyme have indicated that methionine synthase is a modular protein with binding sites for homocysteine, 5-methyltetrahydrofolate, cobalamin, and S-adenosylmethionine. The homocysteine-binding domain has been shown to have the ability to catalyze methyl transfer from cobalamin to homocysteine when expressed alone. Binding of cobalamin to the enzyme causes the cofactor to undergo a dramatic shift in structure because a histidine residue from the protein replaces the dimethyl-benzimidazole nucleotide that is coordinated to the cobalt ion in free cobalamin. The S-adenosylmethionine binding site is essential for function as it interacts with methionine synthase reductase to keep cobalamin in its active +1 oxidation state. Over time, the cob(I)alamin cofactor for methionine synthase becomes oxidized to cob(II)alamin, which makes it unsuitable as a methyl acceptor from 5-methyltetrahydrofolate; as a result, methionine synthase becomes inactive. Regeneration of a functional cofactor requires both methionine synthase reductase and S-adenosylmethionine (Matthews, 1998).

Cloning of methionine synthase reductase showed the value of studying microorganisms for cobalamin metabolism as consensus sequences were used from a reducing system found in bacteria. In *E. coli*, two flavoproteins encoded by two different genes are required for reduction of cobalamin used by methionine synthase: a flavodoxin protein containing FMN and a NADPH-ferredoxin reductase (Fujii and Huennekens, 1974; Osborne et al., 1991). It was predicted that

the human reductase would be a “fusion” of the two systems as only one complementation group represented methionine synthase reductase patients (cblE). Using overlapping sequences homologous to FAD and NADPH-binding domains, TBLASTN searches were performed on EST databases. This yielded a sequence in *C. elegans*, which was used to identify a potential human sequence. The coding sequence of the gene contains 698 amino acids and is found on chromosome 5 p15.2-p15.3 (Leclerc et al., 1998).

Chapter #2
Inborn Errors of Folate Metabolism

There are a number of confirmed inherited disorders of folate metabolism, including 5,10-methyleneTHF reductase deficiency, methionine synthase deficiency (*cblG*), methionine synthase reductase deficiency (*cblE*), and hereditary folate malabsorption. Glutamate formiminotransferase deficiency has also been confirmed and will be discussed in chapter 3 of this thesis. There are also a number of suspect disorders. These include dihydrofolate reductase deficiency and methenyltetrahydrofolate cyclohydrolase deficiency. These diseases are considered suspect as the cases reported in the literature have either been retracted or contain questionable medical evidence.

Hereditary Folate Malabsorption

Characterized by megaloblastic anemia and progressive neurological deterioration, hereditary folate malabsorption is caused by a failure of the gut to effectively take up ingested folate from the diet (Rosenblatt and Fenton, 2001). The original patients with the disorder were two sisters with megaloblastic anemia, mental retardation, convulsions and ataxia (Luhby et al., 1965). Most of the diagnosed patients have presented with a similar phenotype. However, there has also been a report of a patient who did not present with neurological manifestations. This patient was a 4 month old boy who had reoccurring infections due to a deficiency in cellular and humoral immunity. In other patients, increasing serum folate concentrations did not result in an increase in folate concentrations in the cerebrospinal fluid; however, elevation of serum folates in this patient did result in an increase in folate levels in cerebrospinal fluid (Urbach et al., 1987).

Because patients diagnosed with the disease are incapable of absorbing either reduced or oxidized folates, all folates must share the same transport system across the intestinal wall. This suggests that intestinal uptake uses a different transport system than that of cellular uptake. Current treatment regimens include large oral doses of folic acid, folinic acid or 5-

methyltetrahydrofolate; patients who fail to respond to oral administration can receive intramuscular injections of folate in order to bypass the intestinal transport block. Success of treatment is determined by normalization of folate levels in the serum, red blood cells and cerebrospinal fluid (Rosenblatt and Fenton, 2001).

Defects in the Cellular Uptake of Folate

There are a few patients identified with the inability to take up folate into cells. The original patient described with a defect in cellular uptake of folate was a young man with severe aplastic anemia and an extensive family history of leukemia, pancytopenia and neutropenia. Administration of oral folic acid to the patient resulted in a vast clinical improvement. Experiments measuring the uptake of 5-[¹⁴C]methyltetrahydrofolate in stimulated peripheral blood lymphocytes and bone marrow cells showed significant reduction of uptake compared to controls. The deoxyuridine suppression test, intestinal absorption and polyglutamate formation were all normal (Branda et al., 1978). Although this suggests an abnormality in the RFC system, it is interesting to note that his son exhibited a similar abnormality only after presenting with neutropenia. It is possible that this family is presenting something other than a primary defect in folate metabolism (Rosenblatt and Fenton, 2001).

Another reported case involved dyserythropoiesis in a 30 year old man. His marrow and blood cells both showed decreased 5-[¹⁴C]methyltetrahydrofolate uptake, with an overall reduced V_{\max} evident. Interestingly, lymphocytes showed normal uptake levels and folic acid uptake was normal in all cell types. These results suggest an error in the RFC transport system only in erythrocyte and bone marrow precursors (Howe et al., 1979). Overall, the reported patients show varied clinical features and hematological manifestations were not present in all cases. Whether

or not these cases truly represent an inherited inborn error of folate metabolism is still under debate (Rosenblatt and Fenton, 2001).

Dihydrofolate Reductase Deficiency

Dihydrofolate reductase deficiency remains a suspect disorder, with three putative patients described. The first identified patient came to medical attention due to megaloblastic anemia that responded better to folinic acid than folic acid. DHFR enzyme assays from the liver showed only 35% of normal activity. Currently, the patient is still folate-dependent. However, studies of cultured fibroblasts from the patient indicate normal levels of DHFR enzyme activity (Rosenblatt and Fenton, 2001).

The other two patients were siblings originally diagnosed with DHFR deficiency because of an abnormal deoxyuridine suppression test, megaloblastic anemia and response to folinic acid. In addition, liver cell homogenates from the two patients showed no DHFR enzymatic activity until exposed to high concentrations of cations (Tauro et al., 1976). When the cases were reinvestigated because the patients had failed to continually respond to folinic acid, it was determined that they both suffered from a complete absence of TCII, a serum protein essential for cobalamin uptake into cells. Interestingly, their parents showed an intermediate phenotype in that they both had only 50% normal TCII binding activity, suggesting heterozygosity for a null TCII allele (Hoffbrand et al., 1984).

Methenyltetrahydrofolate Cyclohydrolase Deficiency

Three cases have been reported of putative 5,10-methenyltetrahydrofolate cyclohydrolase deficiency. The first patient was a microcephalic 7 month old female presenting with mental retardation. Electroencephalograms indicated that the patient had hypersarrhythmia and pneumocephalographs showed dilation of the lateral ventricles and an enlarged third ventricle.

There was elevated serum folate, but cobalamin levels were determined to be normal. The second patient was a microcephalic 4 month old girl with mental retardation and defective vision. Hypsarrhythmia was observed and there was clear enlargement of lateral ventricles and third ventricle. The patient also presented with significant cortical atrophy. Serum folates were highly elevated, while serum cobalamin was lower than expected. The third patient also had mental retardation and electroencephalograms indicated dysrhythmia. There was cortical atrophy and marked enlargement of the ventricles. Serum folate levels were elevated and cobalamin levels were normal. All three patients excreted normal levels of FIGLU after oral histidine loading and showed marked decrease of cyclohydrolase activity in erythrocytes and liver (Arakawa et al., 1966). In a later review by the primary author, the diagnosis of cyclohydrolase deficiency was withdrawn for unknown reasons (Arakawa, 1970).

Methylenetetrahydrofolate Reductase Deficiency

Methylenetetrahydrofolate reductase deficiency is an autosomal recessive disease which results in the biochemical phenotype of homocystinuria with normal to low levels of plasma methionine in the absence of megaloblastic anemia. Currently, there are over 40 individuals diagnosed with the disease. Clinically, the spectrum is very diverse, with the age of onset and disease features quite variable. The most prominent clinical feature is developmental delay, but cardiovascular disease and loss of motor capabilities has also been observed in the patients (Rosenblatt and Fenton, 2001).

The first patients identified with the disorder were diagnosed in adolescence with clinical features milder than what are usually observed in this disorder. One such case involved a mildly retarded 15 year old female with a 2 year history of psychiatric difficulties. Hyperhomocystinemia and normal levels of methionine were observed in the blood.

Administration of pyridoxine and folic acid helped resolve the psychiatric manifestations. Enzyme assays on cultured fibroblasts and liver extracts showed a deficient methylenetetrahydrofolate reductase and normal cystathionine β -synthase (Freeman et al., 1972). A more severe form of the disease was later reported. One example involves two infants who died within the first year of life due to vascular thrombosis and complications. Methylenetetrahydrofolate reductase deficiency was determined by enzyme assays on peripheral leukocytes from the patients (Narisawa et al., 1977). The disease can also be heterogeneous within families. Two brothers diagnosed with methylenetetrahydrofolate reductase deficiency were similar biochemically but did not share similar clinical phenotypes. The younger brother lost much of his motor capabilities and was wheelchair bound by his early 20s. This was also accompanied by noticeable memory lapses. In contrast, his older brother remained clinically asymptomatic (Haworth et al., 1993).

Diagnosis of the disease can be done using cultured fibroblasts as the enzyme is expressed in all tissue. Studies comparing enzyme activity to growth curves have shown that overall activity increases as the confluency of the culture increases, with the greatest level of activity observed in confluent cells (Rosenblatt and Erbe, 1977). This observation can affect clinical diagnosis if not accounted for. Cells with a defective methylenetetrahydrofolate reductase become auxotrophic for methionine in methionine-depleted media supplemented with folate, cobalamin and homocysteine (Rosenblatt and Erbe, 1977).

Methylenetetrahydrofolate reductase deficiency remains a difficult disease to treat. Patients are usually provided with a regimen that includes folates, methionine, pyridoxine, betaine, cobalamin and carnitine. Folates are given in order to maximize any possible activity that the mutated enzymes may possess. 5-methyltetrahydrofolate is also administered as it is the

product of the reaction. High plasma levels of homocysteine are treated using betaine, a substrate for the liver-specific betaine:homocysteine methyltransferase, and pyridoxine, the essential cofactor for the cystathionine β -synthase. To sustain the active-methyl cycle, methionine is administered along with cobalamin, the essential cofactor for methionine synthase. Carnitine is also administered, as its synthesis requires S-adenosylmethionine which is the methyl donor of the active methyl cycle. Of all the attempted treatments, only betaine administration has made a difference (Rosenblatt and Fenton, 2001).

Common polymorphisms within MTHFR have been associated with increased risk for cardiovascular disease within the general population. Of particular interest is a common 677C \rightarrow T polymorphism. In 1988, a thermolabile variant of MTHFR causing mild hyperhomocystinemia that could be corrected by elevated levels of folate was identified (Kang et al., 1988). The genetic basis for thermolability was 677 C \rightarrow T, resulting in the conversion of a conserved alanine at position 222 to a valine (Frosst et al., 1995). Homozygotes and heterozygotes for the thermolabile polymorphism generally have increased levels of plasma homocysteine and an increased risk for cardiovascular disease, but the levels of plasma homocysteine depend greatly on the folate status of the individual. Initial work found the polymorphism to have a frequency of 38% in chromosomes unselected for ethnicity (Frosst et al., 1995). 677C \rightarrow T may also be a significant risk factor for neural tube defects. Preliminary work found only a mild increase in risk in infants homozygous of the change (Shaw et al., 1998), but further work indicated that the risk becomes much greater when combined with low folate status (Christensen et al., 1999).

Polymorphisms in MTHFR have also been examined for their effect on cancer and Down Syndrome. For colorectal cancer, individuals homozygous for 677C \rightarrow T showed a decreased risk

for the disease if accompanied by low consumption of alcohol (Chen et al., 1996). Another polymorphism, 2756A→G, if in homozygosity also showed a decrease in colorectal cancer risk when accompanied by low consumption of alcohol. This study, however, was performed on a small sample, resulting in large confidence intervals (Ma et al., 1999). Research into prostatic carcinoma found that homozygosity for 677C→T was associated with a higher tumor grade (Kimura et al., 2000). 677C→T has also been associated with an increased risk for cervical intraepithelial neoplasia (Piyathilake et al., 2000).

Mothers heterozygous for 677C→T showed a 2.6-fold higher risk for meiotic dysjunction causing Down Syndrome than mothers without the polymorphism. Further work showed that mothers with children afflicted with Down Syndrome have a significant increase in plasma homocysteine levels and lymphocyte methotrexate toxicity, indicating an imbalance in folate and methionine metabolism (James et al., 1999).

Methylcobalamin Deficiency

Clinically, individuals with defects in methylcobalamin synthesis generally present symptoms within the first year of life and can be regarded as impacting the patient at three levels. Hematologically, the patient suffers from megaloblastic anemia. This generally reflects problems in DNA replication from folate becoming trapped as 5-methyltetrahydrofolate, affecting purine and pyrimidine synthesis. The “folate-trap” hypothesis originated from the observation that individuals with folate deficiency have the same hematologic abnormalities as those with methylcobalamin deficiency (Herbert and Zalusky, 1962). Short term neurological difficulties are also prevalent, which may be due to poor neurotransmitter synthesis because of a shortage of S-adenosylmethionine. Patients with methylcobalamin deficiency also suffer from long term developmental defects due to poor myelination in the nervous system (Stabler, 1999).

There is genetic heterogeneity among patients with methylcobalamin deficiency. It was shown that methionine synthase activity was normal under standard reducing conditions for certain methylcobalamin deficient cell lines and low for other methylcobalamin deficient cell lines under the same conditions. Complementation analysis indicated the presence of the two distinct groups, reflecting mutations at separate loci. As a result, cell lines with methylcobalamin deficiency but with normal methionine biosynthesis under standard reducing conditions were given the designation *cblE* while cell lines with methylcobalamin deficiency but with decreased methionine biosynthesis under standard reducing conditions was designated *cblG* (Watkins and Rosenblatt, 1988). Patients diagnosed with *cblG* disease have errors in methionine synthase, while patients diagnosed with *cblE* have errors in methionine synthase reductase.

There are a number of *cblG* mutations reported in the literature. P1173L, Δ Ile881 and H920D were reported with the cloning of the gene (Li et al., 1996; Leclerc et al., 1996). Mutations have also been identified in patients who are marked by the complete absence of methionine synthase mRNA (Wilson et al., 1998). Further exploration into the P1173L mutation has shown that it is present in 10 of 21 *cblG* patients, suggesting that it is a common mutation causing the disease (Rosenblatt et al., 2000). It has been hypothesized that common polymorphisms in methionine synthase that raise the levels of homocysteine may be risk factors for cardiovascular disease analogous to what is observed in MTHFR; however, none has been identified to date.

There have been a number of reported mutations in patients suffering from *cblE* disease. The first reported mutations involved a 4 bp frameshift in two affected siblings and a 3 bp deletion in an unrelated patient (Leclerc et al., 1998). Further mutation analysis of *cblE* patients identified 11 novel mutations in 8 patients. Of the mutations reported, there were errors to splice

junctions, small deletions and point mutations (Wilson et al., 1999). There has also been the discovery of a polymorphism, 66A→G, that is associated with an increased risk for spina bifida if either the mother or child is homozygous. The risk appears to be substantially increased in the presence of low levels of cobalamin (Wilson et al., 1999).

Methylcobalamin deficiency is diagnosed by low incorporation of label from [¹⁴C]methyltetrahydrofolate into intracellular protein (Gravel et al., 1975). Hydroxocobalamin has been shown to resolve the biochemical abnormalities of the disease and partially resolve the developmental and neurological abnormalities. Success of these treatments depends on how early therapy is commenced. Hematological abnormalities can also be corrected with folate supplementation. Betaine and pyridoxine have also been administered to help control the high levels of homocysteine that mark the disease (Rosenblatt and Fenton, 2001).

Chapter #3
Biochemistry of Glutamate Formiminotransferase-Cyclodeaminase
&
Glutamate Forminotransferase Deficiency

Degradation of histidine is a source of one carbon units for folate metabolism, with the breakdown intermediate N-formiminoglutamate being acted upon by glutamate formiminotransferase-cyclodeaminase, a folate-dependent enzyme. N-formiminoglutamate is generated by the following metabolic pathway:

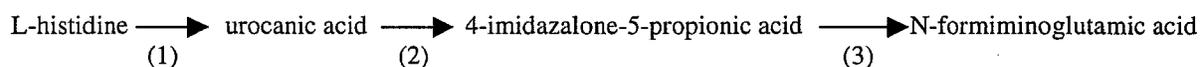


Figure #4: Breakdown of L-histidine to N-formiminoglutamic acid. 1) L-histidine is broken down to urocanic acid by histidase, an enzyme which can be induced by glucagon. 2) Urocanic acid is broken down to imidazole-5-propionic acid by urocanase. 3) 4-imidazole-5-propionic acid is broken down to N-formiminoglutamic acid by imidazole-5-propionate aminohydrolase (Shane and Stokstad, 1986).

The overall reaction for glutamate formiminotransferase-cyclodeaminase (FTCD) results in the production of glutamate, NH_3 , and 5,10-methylenetetrahydrofolate from each molecule of N-formiminoglutamate. The occurs in two steps and is given in figure #5:

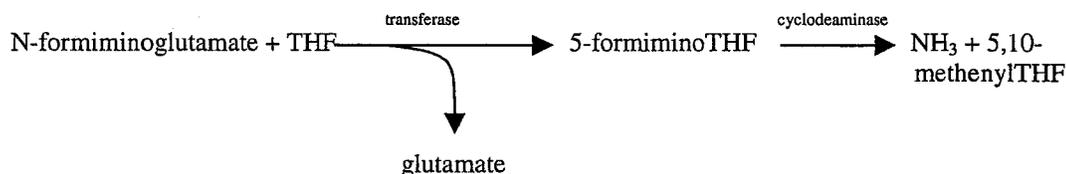


Figure #5: The two reactions catalyzed by glutamate formiminotransferase-cyclodeaminase.

Enzymatic activity *in vivo* is affected by glucagon, epinephrine and insulin levels. Glucagon and epinephrine cause a rapid increase in hepatic formiminotransferase activity while insulin causes a decrease in activity. Since these three hormones are involved in regulating blood glucose levels and since the breakdown of histidine generates precursors for gluconeogenesis, it is possible that glutamate formiminotransferase-cyclodeaminase plays a more important role in controlling histidine levels and providing substrates for gluconeogenesis than in generating one-carbon units for folate metabolism (Weisenberg, 1972).

An alternative reaction for the formiminotransferase reaction has been reported. Using N-formylglutamate and THF, the formiminotransferase is capable of generating 5-CHO-THF and glutamate (Bortoluzzi and Mackenzie, 1986). N-formylglutamate was also shown to be a competitive inhibitor of the formiminotransferase reaction and has a significantly higher affinity for the enzyme than N-formiminoglutamate.

Microorganisms do not possess the glutamate formiminotransferase enzyme. Instead, the N-formylglutamate reaction is the primary reaction, with N-formiminoglutamate is hydrolyzed to ammonia and N-formylglutamate first before generating the one-carbon unit for folate metabolism. Some microorganisms, however, possess the cyclodeaminase enzyme. The cyclodeaminase breaks down 5-formiminotetrahydrofolate produced by the formiminoglycine formiminotransferase reaction of the purine degradation pathway. The bacterial cyclodeaminase has been purified from *Clostridium cylindrosporum*. The enzyme's MW is 38,000 Da and is strongly inhibited by THF. The enzyme is optimally active at pH 7.2 and is capable of catalyzing the reverse reaction at pH 8.2 (Uyeda and Rabinovitz, 1967).

It has been shown that *Dirofilaria immitis*, a type of filaria, and *Brugia pahangi*, a type of nematode, possess both formiminotransferase and cyclodeaminase activities. Like the mammalian version of the enzyme, it is a bifunctional octamer consisting of eight identical subunits. There are differences between the filarial and mammalian forms in response to pharmacological agents. Diethylcarbamazine citrate, an anti-filarial agent, is a potent inhibitor of the filarial but not the mammalian form of the enzyme. The antimalarial agent pyrimethamine has also been shown to inhibit the filarial cyclodeaminase (Jaffe et al., 1980).

Protein Structure, Biochemistry and Enzymology

Protein studies on mammalian glutamate formiminotransferase-cyclodeaminase have been done primarily on extracts from hog liver. The initial purification of glutamate formiminotransferase was from acetone treated porcine liver and it was found that the cyclodeaminase copurified with it. This suggested that both activities were present on a single peptide. Current purification protocols from hog liver involve a similar approach but do not use acetone to dissolve the cell membranes. This greatly increases the yield of enzyme and prevents unnecessary chemical modification of the protein (Drury et al., 1975).

Full length porcine FTCD is a monomer with a molecular weight of 62 kDa. Isoelectric focussing in 8 M urea and cyanogen bromide treatment demonstrated that FTCD contains only identical subunits. Electron microscopy shows that octameric FTCD is arranged as a tetramer of dimers and forms a planar ring. Kinetic observations and conditions to selectively destroy each of the catalytic activities indicate that both reactions are not part of a single active site. Formation of the planar ring structure is essential for both catalytic functions (Beaudet and Mackenzie, 1976).

Treatment of purified enzyme with the sulfhydryl reagent 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) has been shown to selectively inactivate the cyclodeaminase by modifying two different cysteine residues. The first cysteine is rapidly modified with no effect on catalytic function, indicating that it is a non-essential residue. The second cysteine is modified slowly and with elimination of enzyme activity. Slow modification of the residue suggests that it is protected by the active site of the enzyme and loss of activity indicates that it is a catalytic residue. The effects of DTNB can be reversed by DTT, proving that loss of catalytic activity is not due to denaturation of the protein (Drury and Mackenzie, 1977).

Channeling of substrate was investigated by supplying polyglutamated substrates for the formiminotransferase and observing the time of conversion between 5-formiminotetrahydrofolate and 5,10-methenyltetrahydrofolate. If channeling existed between the two catalytic activities, then any 5-formiminotetrahydrofolate generated by the formiminotransferase should be converted almost instantaneously to 5,10-methenyltetrahydrofolate. If there was no channeling, then 5-formiminotetrahydrofolate should build up in solution until it reaches a sufficient concentration to bind to the enzyme via diffusion. Since production of 5,10-methenyltetrahydrofolate was almost instantaneous, it was concluded that substrate channeling occurred between the active sites for the formiminotransferase and cyclodeaminase (Mackenzie and Baugh, 1980).

More about the nature of substrate channeling between the two active sites was revealed through the use of chymotrypsin. Cleavage with the protease inactivates the cyclodeaminase, but leaves formiminotransferase activity intact. FTCD modified with chymotrypsin maintained the same K_m for monoglutamate substrates, but specificity for polyglutamate substrates was lost. These results suggest that the bifunctional enzyme may have only one polyglutamate chain-binding site for each formiminotransferase and cyclodeaminase unit (Mackenzie et al., 1980). This conclusion was confirmed by saturating the transferase with tetrahydrofolate-polyglutamate, then providing 5-formiminotetrahydrofolate with various polyglutamate lengths for the cyclodeaminase. The cyclodeaminase was only capable of converting 5-formiminotetrahydrofolate to 5,10-methenyltetrahydrofolate if the folate molecule was monoglutamate rather than polyglutamate. This showed that the polyglutamate site was saturated by the tetrahydrofolate, and that monoglutamates were the only forms that could diffuse into the active site of the cyclodeaminase from solution. It was determined that the

octamer is capable of binding four molecules of tetrahydrofolate, one per bifunctional pair. Efficiency of channeling was also the highest when the glutamate chain was five units long. Oversized chains are less effective at channeling because of steric hinderance due to their larger size. Undersized chains could not participate in channeling because they were not long enough to “swing” between the two active sites (Paquin et al., 1985).

Characterization of the bifunctional unit started by denaturation experiments by varying the concentrations of urea. As concentration of urea increases, the bifunctional octamer breaks down into functional dimers followed by inactive monomers. In 1 M urea and THF, the solution retains formiminotransferase activity while in 3 M urea and THF, the solution retains cyclodeaminase activity. Proteolysis of both solutions with chymotrypsin produces different fragment patterns on protein gels. These results demonstrate that one dimeric interface is responsible for formiminotransferase activity, while another dimeric interface is responsible for cyclodeaminase activity. Only when the dimers form the native octamer is there channeling of substrate (Findlay and Mackenzie, 1988).

The FTCD protein was split into its individual functional domains by subcloning into expression vectors only the sequence for each domain. Isolated domains show self-dimerization; however, if isolated domains are put into solution together, self-dimerzation occurs but no bifunctional octamer forms. This illustrates the importance of the linker region in maintaining domain interactions within each subunit of the octamer (Murley and Mackenzie, 1995). The various oligomeric forms possible for FTCD are shown in figure #6:

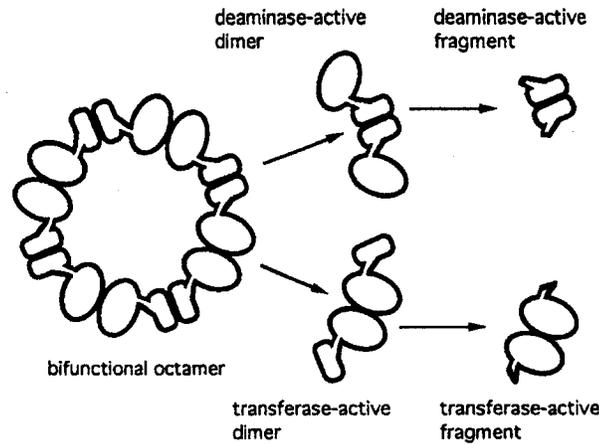


Figure #6: Catalytically active forms of glutamate formiminotransferase-cyclodeaminase. The enzyme can either catalyze both reactions when in the bifunctional octamer form, or catalyze the individual reactions as a transferase or cyclodeaminase dimer. Note that the functional domain is all that is required for the individual reactions, so truncated proteins missing the other functional domain still have catalytic activity as long as dimerization can occur (Murley and Mackenzie, 1996).

The crystal structure of the formiminotransferase domain has been elucidated. Each monomer is made up of N and C terminal subdomains. Each subdomain consists of a β -sheet with α -helices located on the external surface. The binding site for 5-formiminotetrahydrofolate lies between the two subdomains of a monomer with the substrate making extensive contacts with residues in both (Kohls et al., 2000).

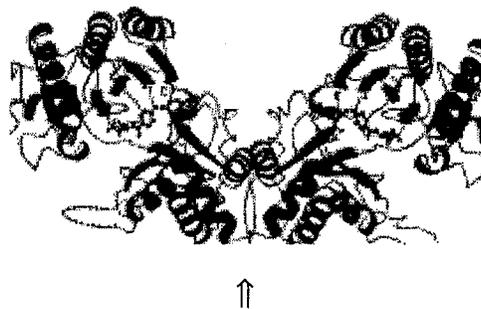


Figure #7: Ribbon representation of formiminotransferase domain as a dimer. The dimerization domain is located by the arrow, with the α -helices and the loops being critical parts (Kohls et al., 2000).

The dimer interface is important as dissociation of the dimer results in a loss of catalytic activity. The dimer interface exists in the C-terminal subdomain. Three loop regions as well as

a C-terminal α -helix make hydrogen-bonding contacts as well as hydrophobic interactions within the dimerization site. The C-terminal helix is of great importance as it possesses a polar face which makes hydrogen-bonding contacts with the loop regions of the other monomer (Kohls et al., 2000).

A catalytic mechanism for the transferase reaction has been proposed. Within the active site, it is suggested that His82 extracts a proton from N⁵ of tetrahydrofolate, thus increasing the nucleophilicity of the molecule. This would facilitate the nucleophilic attack of the imino carbon atom of formiminoglutamate. The protonated His82 could then facilitate the breakdown of the intermediates by protonating the amino group of glutamate, thus producing the products. Although this mechanism is supported by the enzyme structure, site-specific mutagenesis has not been performed to confirm it (Kohls et al., 2000).

Cloning and Expression Pattern for Human Glutamate Formiminotransferase

The gene encoding human FTCD was discovered by examination of ESTs on chromosome 21 as part of a study assessing the molecular bases of Down Syndrome. ESTs were compared to the previously identified porcine version of FTCD. Human FTCD maps to 21q22.3 and the major cDNA encodes a protein containing 541 amino acid residues and has 84% homology with the porcine version (Solans et al., 2000; Murley and Mackenzie, 1995). Screening of a human kidney cDNA library indicated that at least five different FTCD transcripts exist within the cell. The two most frequently found transcripts are of lengths 1875 bp (form A) and 1900 bp (form B). Both encode for the same protein but differ in their 3' UTRs. FTCD form C is a result of a 20 bp deletion at c1584, producing a 572 amino acid protein. Form D is created by a 44 bp deletion at c1304, which produces a 495 amino acid protein. Form E is created by a 25 bp insertion at c411, creating a protein of 158 amino acids in length. The

physiological significance for the different transcripts is currently unknown, although not all transcripts were observed in the liver, suggesting some form of developmental regulation. Multiple tissues were screened with an FTCD probe to determine the tissue expression of the protein. FTCD mRNA was detected at the highest levels in the liver, with kidney and testis showing lower levels of transcription. These results suggest that enzymatic studies of the protein should be performed on liver extracts (Solans et al., 2000).

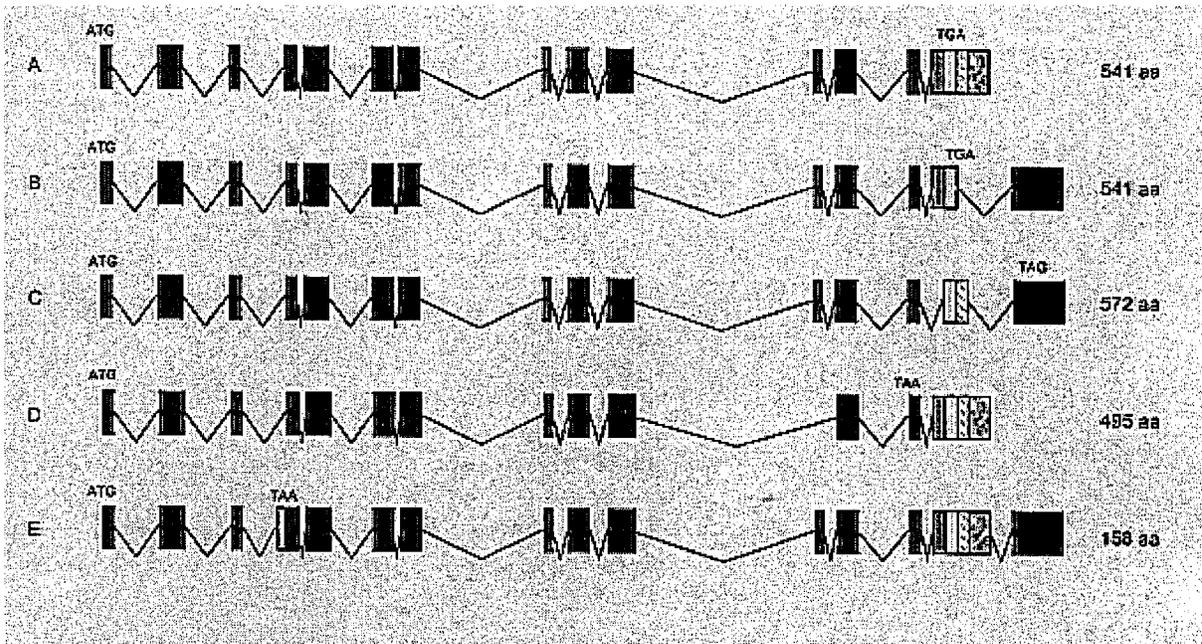


Figure #8: Splicing Variants for FTCD. The five major splicing variants for human FTCD. Protein size and site of splicing variation is indicated. Transcripts 3-5 have major frame shifts, with its significance unknown (Solans et al., 2000).

Glutamate Formiminotransferase Deficiency

Glutamate formiminotransferase deficiency is found in both males and females, and is inherited in an autosomal recessive manner. In an ideal situation, diagnosis of the deficiency is confirmed by the presence of high levels of formiminoglutamate (FIGLU) in the urine and low activity of the enzyme from liver biopsies (Erbe, 1986). Enzyme assays are recommended because high FIGLU excretion may occur due to folate or methylcobalamin deficiency. It has

been proposed that red blood cells can be used for the assay (Arakawa et al., 1966), but expression analysis indicates that erythrocytes do not express FTCD mRNA (Solans et al., 2000) and Niederwieser and colleagues in 1974 reported that attempts to assess erythrocytes for enzyme activity were unsuccessful. To date, there have been fewer than 20 reported cases of FTCD deficiency, of which very few have had enzyme activity assessed (Erbe, 1986). As an alternative to urine FIGLU assays, hydantoin-5-propionate can also be used as a marker for FTCD deficiency. This compound is generated by the oxidation of 4-imidazolone-5-propionic acid, a precursor to 5-formiminoglutamate (Niederwieser et al., 1976).

There is a great deal of heterogeneity in the levels of FIGLU excreted by patients with glutamate formiminotransferase deficiency. Abnormally large amounts of FIGLU have been detected in some patients on a normal diet, while in other patients abnormal FIGLU excretion was observed only in response to histidine loading (Erbe, 1986). Levels of FIGLU in the urine can also vary depending on the age of the patient, as infants have a much higher excretion of FIGLU following the administration 0.33 g/kg body weight of L-histidine monochloride than older children (Arakawa et al., 1965).

Glutamate formiminotransferase deficiency was first identified in patients of Japanese descent. In 1963, a female patient was brought to medical attention within the first year of life due to mental retardation. Serum folate levels were highly elevated and FIGLU was found in the urine following histidine loading. A liver biopsy and subsequent enzyme assay showed that FTCD activity was about 33% of normal (Arakawa et al., 1963). A second case came to medical attention in 1965 when a female patient was identified with mental and physical retardation. A positive ferric chloride test on urine samples identified the presence 4-amino-5-imidazolecarboxamide (AICA), suggesting a disturbance of purine biosynthesis. Bone marrow

was not megaloblastic, and urine samples tested positive for FIGLU in the absence of histidine loading. The patient died of bronchopneumonia at the age of 1 year 7 months. A post-mortem liver biopsy was performed, with post-mortem liver biopsies from a normal 9 month old child and a normal adult serving as controls. Depending on the time the enzyme assay was halted, the patient's enzyme possessed between 30-50% activity of the controls, with higher activities observed in the longer assays (Arakawa et al., 1965). The third case involved a female patient who came to medical attention due to mental retardation. Electroencephalograms showed sporadic spikes and sharp waves in the bilateral fronto-parieto-temporal areas, suggesting diffuse brain damage. Serum folate activity was found to be highly elevated. Urinary FIGLU was found to be elevated following histidine loading. Unlike the first two patients, enzyme assays were performed on erythrocyte lysates. These experiments indicated that her enzyme possessed between 15-35% of the activity exhibited by the controls. Erythrocyte lysates from the first case were also examined, and the values were very similar to what was observed from the liver biopsies. (Arakawa et al., 1966). Based on the initial clinical features of the patients, glutamate formiminotransferase deficiency was defined by mental retardation, hyperfolcic acidemia and FIGLU excretion in response to administration of L-histidine monochloride.

Following diagnosis of the original cases, other patients were identified that expanded the clinical spectrum of the disease. These patients were not of Japanese descent and presented a very different phenotype than those originally defined. In 1974, two sisters were identified with glutamate formiminotransferase deficiency. They excreted 10 times as much FIGLU in response to the histidine loading protocol compared to those patients identified by Arakawa. Both patients also had normal serum folate levels and no hematological abnormalities. Mild mental retardation occurred only in one of the two sisters. Attempts to assess enzyme activity on erythrocyte

lysates proved unsuccessful, and no liver biopsies were performed (Niederwieser et al., 1974). Other cases include two American siblings who excreted very high levels of FIGLU in the absence of histidine. Both presented with normal serum folate levels, no hematological findings and no mental retardation. No enzyme assays were performed on either liver or erythrocyte lysates (Perry et al., 1975). There has also been one reported case of a deficient FTCD being diagnosed in adulthood. An adult white female of age 42 was identified with the deficiency after coming to attention due to faulty carbohydrate metabolism. She responded to folate supplements and a decrease in dietary carbohydrate. No enzyme assay was performed (Herman et al., 1969).

Glutamate formiminotransferase deficiency is also clinically heterogeneous within families. Two affected brothers of Japanese descent were determined have the deficiency, and both presented different clinical features. The first child was diagnosed with pyridoxine-folic acid responsive megaloblastic anemia 15 days after birth. His diagnosis was changed to glutamate formiminotransferase deficiency due to FIGLU excretion in response to histidine loading, AICA excretion, and abnormally high serum folate levels. Severe mental retardation was apparent and electroencephalograms showed low voltage in the frontal regions at 1 ½ years of age. Enzyme assays performed on a liver biopsy showed 55% activity compared to a control (Arakawa et al., 1968). His brother was diagnosed at 6 months of age after a histidine loading test, but presented with a considerably different phenotype. He did not have megaloblastic anemia and although there was cortical atrophy of the frontal and parietal regions, there was no obvious mental retardation. No enzyme assay was performed. The parents were also examined, and they presented with elevated urinary FIGLU after histidine loading, slight macrocytosis, and abnormal electroencephalograms (Arakawa et al., 1968). With the parents also presenting

abnormal clinical features, it is difficult to determine which features of the patients are actually associated with the deficiency.

Other branches of folate metabolism have been examined for activity in some glutamate formiminotransferase deficient patients. Tetrahydrofolate-dependent enzymes were checked in erythrocyte lysates from two patients of Japanese descent. 5,10-methylenetetrahydrofolate dehydrogenase and serine hydroxymethyltransferase showed elevated levels of activity in patients versus controls, while 5,10-methenyltetrahydrofolate cyclohydrolase activity remained at normal levels (Arakawa et al., 1966). Purine biosynthesis has also been examined when AICA was found to be excreted in the urine of an infant with glutamate formiminotransferase deficiency. AICA is converted by the cell to AICAR, a compound involved in purine biosynthesis. After oral administration of AICA, two glutamate formiminotransferase deficient patients excreted more AICA in the urine than control individuals (Arakawa and Wada, 1966). Subsequent enzyme assays of AICAR transformylase from erythrocytes from 5 individuals with glutamate formiminotransferase deficiency showed levels of activity to be within the normal range (Arakawa and Hirono, 1966). A patient of Japanese descent also presented with defective glycine incorporation into urinary uric acid (Arakawa et al., 1972).

Based on the reported cases, patients appear to present with either a severe or mild phenotype. Of particular interest is the variation in the amount of urinary FIGLU excreted between severely and mildly affected patients. Urinary FIGLU levels for patients with glutamate formiminotransferase deficiency is shown in table #1. It should be noted that unaffected individuals do not excrete FIGLU in the urine.

Table #1: FIGLU excretion levels for patients diagnosed with glutamate formiminotransferase deficiency.
(nr=not reported)

Patient	Phenotype	FIGLU in urine ($\mu\text{mol}/24\text{ h}$) on normal diet	FIGLU in urine ($\mu\text{mol}/24\text{ h}$) after histidine loading
CK (Arakawa et al, 1963)	severe	nr	40-200
HK (Arakawa et al., 1965)	severe	768	nr
YA (Arakawa et al., 1968)	severe	nr	258-6279
YN (Arakawa et al., 1968)	severe	nr	205-262
FD (Niederwieser et al., 1974)	mild	1470-3320	10700
LD (Niederwieser et al., 1976)	mild	3150	8220
KS (Perry et al., 1975)	mild	3494-3897	nr
LS (Perry et al., 1975)	mild	2833	nr
WG 1795 (Beck et al., 1981)	mild	1700	nr
- (Russell et al., 1977)	mild	720-1000	7000

In general, patients who present with the mild form of the disease excrete high levels of FIGLU in the absence of histidine loading, while severely affected individuals excrete low levels of FIGLU under the same circumstances. In addition, urinary FIGLU levels were much higher after histidine loading in patients with the mild form of the disease compared to severely affected individuals. Biochemically, these observations could be explained as two different enzymatic defects; blocks in the formiminotransferase reaction would result in all FIGLU generated being excreted in the urine, while blocks in the cyclodeaminase will have some of the generated FIGLU being converted to 5-formiminotetrahydrofolate, thus reducing the amount of FIGLU observed in the urine (Rowe, 1983). Based on the biochemical observations, it was postulated that the mild phenotype was due to blocks in the formiminotransferase reaction, while the severe phenotype was due to blocks in the cyclodeaminase reaction (Rowe, 1983). To date, no enzymatic assays have confirmed or disproved this hypothesis.

Whether the severe phenotype exists is currently under debate. Because of ascertainment bias in the study of Japanese patients and the milder phenotypes of non-Japanese patients, Erbe has argued that mental retardation is not a clinical feature of the disease (Erbe, 1986). Some of

the severe patients were confirmed to have the disease using enzyme assays for FTCD using erythrocyte lysates, a cell type which does not express FTCD. In addition, enzyme assays from liver biopsies of severely affected patients show levels of activity much higher than what would be expected from a severe disorder. It is also difficult to explain biochemically the elevated serum folate levels in severely affected individuals; since histidine degradation represents only a small aspect of folate metabolism, it is hard to see how it could create a significant block (Erbe, 1986). Further challenging the existence of a severe phenotype is the lack of severely affected patients who are not of Japanese origin. In Germany, there was a report of a patient presenting with the severe phenotype, with the clinical features being mental and physical retardation as well as orotic aciduria, homocystinuria and FIGLU excretion (Shin et al., 1986). Further studies of the patient revealed that she had been misdiagnosed, and actually was afflicted with *cbIG* disease (Watkins and Rosenblatt, 1989).

If the Japanese patients are not considered, the clinical features for glutamate formiminotransferase become much more uniform. The nine non-Japanese patients summarized in Erbe's review suffered from slight developmental delay, mostly seen as slowed speech development and hypotonia. All of the patients were diagnosed later in life. None suffered from macrocytosis or megaloblastic anemia. These features probably represent the typical features of the disease (Erbe, 1986).

The amount of urinary FIGLU is the major basis for determining whether a therapeutic response has occurred. Folic acid and folinic acid have produced a response in two out of eight patients treated, while one out of two patients responded to oral methionine. Responses to therapy clearly vary and without a full understanding of the clinical phenotype, it is difficult to design effective new approaches (Erbe, 1986; Rosenblatt, 2001).

Chapter #4
Materials and Methods

Experimental Samples

Fibroblasts from three putative patients with glutamate formiminotransferase deficiency were obtained from the Repository for Mutant Human Cell Strains, Department of Biochemical Genetics, Montreal Children's Hospital, Canada. The first two patients, whose cultured cell lines are designated WG 1758 and WG 1759, were siblings diagnosed with glutamate formiminotransferase deficiency. The younger sibling (WG 1758) first came to medical attention at age 2¾ years due to delays in speech development. Column chromatography identified high levels of FIGLU in the urine. At age 3¼ years, he showed signs of physical retardation, with both his height and weight being one standard deviation below average. Other than general hypotonia, the patient presented no neurologic abnormalities, and electroencephalograms were normal. No liver biopsy was performed to confirm his diagnosis. His elder sister (WG 1759) was identified with glutamate formiminotransferase deficiency at age 8 years when the whole family was studied clinically following the diagnosis in the younger brother. She had developed normally at the time of diagnosis. Like her brother, she was hypotonic and considered clumsy. Unlike him however, her electroencephalograms were abnormal and suggested some subcortical dysfunction, although at the time of diagnosis there was no history of seizures. She also excreted very high levels of FIGLU in the urine. No liver biopsy was performed (Perry et al., 1975).

The third patient (WG 1795) was diagnosed at age 2 with glutamate formiminotransferase deficiency. During his first week of life, the boy exhibited two minor apnoeic spells. From age 3 months on, he suffered from recurring otitis media and from age 4 months on, recurring pneumonias. At the time of diagnosis, the boy appeared to be mildly mentally retarded, had breathing difficulties, and was hypotonic. His electroencephalograms and

karyotype were normal. The patient had normal serum folate levels and normal red blood cells, although leukocytes were considered to be slightly hypersegmented. Urinalysis indicated the presence of high levels of FIGLU (Beck et al., 1981).

All three patients are considered to have presented with the mild form of the disease.

DNA Extraction

Fibroblasts from each patient was grown to confluency in one 175 cm² tissue culture flasks using Eagle's Modified Minimal Essential Media (Gibco Life Technologies™, Grand Island, NY, USA) supplemented with 10% fetal calf serum (Wisent Inc., St. Bruno, PQ, Canada). Cells were extracted from each flask by trypsinization, pelleted by centrifugation and washed twice with phosphate buffered saline. Cell pellets were kept at -80°C until the DNA was extracted.

Extraction of DNA was performed via the following protocol. First, each pellet was placed in 0.5 ml of cell suspension solution (10 mM Tris-HCl, 0.4 M NaCl, 2 mM EDTA) in a 1.5 ml Eppendorf tube and mixed until completely suspended. To each suspension, 30 µl of 10% SDS was added to lyse the cells. To remove RNA contamination, each sample was incubated for 15 minutes at 55°C with 10 U of RNase (Hoffman-LaRoche, Laval, PQ, Canada). To free the DNA from bound proteins, each tube was incubated overnight at 47°C after adding 625 µg of proteinase K (Merck, Whitehouse Station, NJ, USA). In addition, SDS is essential for proteinase K function.

Following the overnight incubation, 500 µl of pH 7.5 buffer-saturated phenol (EM Science, Hawthorne, NY, USA) was added to each tube and mixed gently for 5 minutes. This mixture was then spun at 13,000 rpm for 4 minutes in an Eppendorf 5415C centrifuge (Brinkmann Instruments, Mississauga, Ont., Canada). The upper aqueous phase was removed

and placed in a clean 1.5 ml Eppendorf tube. 500 µl of chloroform (Fisher Scientific, Nepean, Ont., Canada) was added and the samples were rotated for 5 minutes. This mixture was then spun at 13,000 rpm for 4 minutes in the Eppendorf 5415C centrifuge. Following centrifugation, the aqueous phase was again removed and placed in a clean 1.5 ml Eppendorf tube. To this solution, 1 ml of cold 95% ethanol was added to precipitate the DNA. The precipitated DNA was spooled onto a sterile capillary and air-dried to remove all traces of ethanol. The precipitated DNA was then resuspended 200 to 300 ul of sterile, deionized water, depending on the size of the precipitate. Quality and quantity was then determined by spectrophotometry at 260 and 280 nm.

Amplification of Exons from Genomic DNA

The FTCD gene consists of 15 exons over a stretch of 19.3 kB of genomic DNA. Each exon was located in the genomic sequence (NCBI accession number 7768685) and primers flanking each exon were designed. When possible, primers were designed to include two exons. Primers and amplification protocols used are given in table #2:

Table #2: Primers and PCR conditions used in the amplification of the FTCD exons.

Exon	Forward Primer (5'-3')	Reverse Primer (5'-3')	Amplification Protocol	[MgCl ₂] (mM)	Annealing Temp (°C)
1	tgtgccttgctggtaaatga	gccttagccactcaagctg	PE Biosystems	2.5	59
2	cctccaagctccttttctt	ccaggacaccaggacagg	PE Biosystems	1.5	61
3	aggacagagggtccgagaac	agggaactgggggatacaga	PE Biosystems	1.5	61
4-5	cgctctccaggaactgag	aggctctcagcctctaacc	Qiagen	1.5	55
6	atgcaagtgccaggagagac	cctggctggaggatgtgg	PE Biosystems	1.5	63
7	gctcaggtgtccaccaatct	aggcaagaccaagcagaaag	PE Biosystems	1.5	61
8	cgcaaaagcacctgtgtg	actcagccgggtctccac	PE Biosystems	2.5	58
9	gcaggttctcagccgagtgg	aacaactgcgcctcccctg	Qiagen	1.5	62
10	tgcgacccccacatccac	aggctgacccgcttctactg	PE Biosystems	2.5	65
11-12	cttgctacagccccttgtg	gtgctctccagcaactcagc	PE Biosystems	2.5	59
13-14	gtcaggcagagaccctcctt	ccccacagcaacaagctg	PE Biosystems	2.5	58
15	attcagaggacctggggttc	tctctcagtgctgactaaca	PE Biosystems	2.5	60

PCR performed using PE biosystems protocols used 1X GeneAmp™ PCR Buffer (Perkin Elmer, Branchburg, NJ, USA) supplemented with the appropriate amount of MgCl₂ from a stock

solution of 25 mM MgCl₂ (Perkin Elmer), 200 µM of dNTPs (Perkin Elmer), and 20 ng of DNA template. 2.5 U of Amplitaq Gold™ (Perkin Elmer) was added to each reaction. The thermocycler program used for the PE biosystems protocol was as follows:

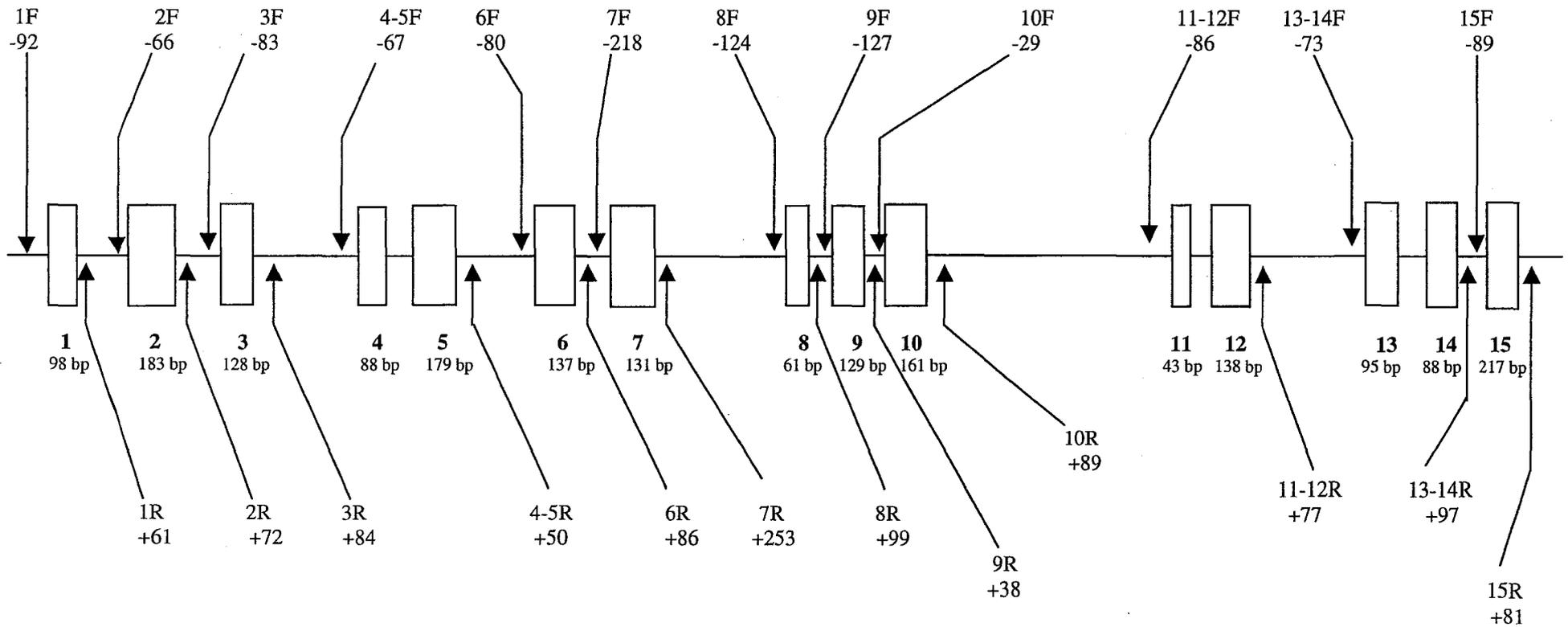
- 1) 96°C for 10 minutes.
- 2) 96°C for 30 seconds.
- 3) Annealing temperature for 30 seconds.
- 4) 72°C for 45 seconds.
- 5) Steps 2-4 repeated 35 times.
- 6) 72°C for 10 minutes

PCRs performed using Qiagen protocols contained 1X HotStarTaq™ PCR buffer (Qiagen, Mississauga, Ont., Canada), 200 µM of dNTPs (Perkin Elmer), and 20 ng of DNA template. 1.0 U of HotstarTaq™ (Qiagen, Mississauga, Ont, Canada) was added to each reaction. The thermocycler program used for the Qiagen protocols were as follows:

- 1) 96°C for 15 minutes.
- 2) 96°C for 30 seconds.
- 3) Annealing temperature for 45 seconds.
- 4) 72°C for 1 minute.
- 5) Steps 2-4 repeated 35 times.
- 6) 72°C for 10 minutes.

The overall schematic of this design is shown in figure #9:

Figure # 9: Genomic structure and location of PCR primers
 (numbers under primer indicate base pair location upstream or downstream from exon splice junction)
 Overall Size of Genomic Sequence: 19 291 bp
 (F=forward primer, R=reverse primer)



Sequencing of Exons

Prior to sequencing, each PCR product was purified using the Multiscreen™ PCR purification plate (Millipore, MA., USA). Sequencing was done using Big Dye Terminator™ Sequencing kit version 2 (Perkin Elmer). Each sample containing 1.0 µl Big Dye Terminator™ mix, 1.5 µl of 5x Big Dye Terminator sequencing buffer, 5.0 µl of water, 0.5 µl of 20 µM primer and 2.0 µl of PCR product. The sequencing reaction was then carried out under the following conditions:

- 1) 96.0 °C for 30 sec.
- 2) 50.0 °C for 15 sec.
- 3) 60.0 °C for 4 min
- 4) Steps 1-3 were repeated 40 times.

Following the sequencing reaction, the products were prepared for sequencing analysis. 30 µl of ddH₂O and 60 µl of 100% isopropanol were added to each sample, followed by incubation of 15 minutes at room temperature. The precipitated products were then pelleted by centrifuging at 3000 x g for 30 minutes. Following removal of the supernatant, each pellet was rinsed with 150 µl of 70% ethanol. The ethanol was then removed, and the samples were recentrifuged at 700 x g for 1 minute. Excess ethanol was evaporated, and the pellet resuspended in 10 µl of formamide. Analysis was performed using the ABI 3700 capillary sequencing system (Perkin Elmer). Data was processed using ABI Prism™ Sequence A software, version 1.12 (Perkin Elmer). Sequence comparisons were made using Autoassembler™ version 2.11 (Perkin Elmer).

Restriction Cuts To Assess Mutation Frequency in 100 Control Individuals

Sequence changes detected by sequence analysis were confirmed by restriction endonuclease analysis of PCR products from 100 control genomic DNA samples.

For c940 G→C mutation, located in exon 7, amplified fragments were cut with AlwI (New England Biolabs, Mississauga, Ont., Canada). Each restriction enzyme reaction contained 3 µl of 10X NEB restriction enzyme buffer #4 (New England Biolabs) 10 µl of PCR product, 1 U of AlwI and 16.5 µl of H₂O for a total volume of 30 µl. Each sample was incubated for 3 hours at 37°C, then electrophoresed in a 1.5% agarose gel at 200V for 1.5 hours on a BRL Model H4 apparatus (Bethesda Research Laboratories, Gaithersburg, MD, USA) . Since the mutation results in the loss of a restriction site, mutant alleles produce a band 557 bp in length while wild-type alleles produce two bands 340 bp and 263 bp in length.

For c1033insG mutation, located in exon 9, amplified fragments were cut with Eco0109I (New England Biolabs). Each restriction enzyme reaction contained 2 µl of 10X NEB restriction enzyme buffer #4 (New England Biolabs), 5 µl of PCR product, 10 U of Eco0109I enzyme, 100 µg/ml of bovine serum albumin, and 20 µl of H₂O. Each sample was then incubated at 37°C overnight, then run on a 2% agarose gel for 1.5 hours at 200V on a BRL Model H4 electrophoresis apparatus (Bethesda Research Laboratories). Eco0109I recognizes the mutant allele, so wild-type alleles will produce two bands 248 bp and 49 bp in length while mutant alleles produce three bands 150 bp, 99 bp, and 48 bp in length.

For c457 C→T, an artificial HinFI site was created to assess the presence of the mutation. The primers used in creating the artificial site are as follows, with the base creating the artificial site in capitals:

c457 C→T forward: 5'-ggcagccaggatggaGagt-3'

c457 C→T reverse: 5'-cttagggagggcctctact-3'

DNA from 100 normal individuals was amplified using the Qiagen protocol described above with an annealing temperature of 55°C, 2% DMSO and 250 ng of DNA template. 10 µl of each

sample was digested by 10 U of HinFI (New England Biolabs) in NEB buffer #2 (New England Biolabs) in a total reaction volume of 20 µl. Cut fragments were separated using a 10% acrylamide (29:1 acrylamide/bis-acrylamide ratio) (BioRad, Mississauga, Ont., Canada). Gels were run for 90 minutes at 70 V in a Mini-Protean II apparatus (BioRad). Wild-type alleles are cut by HinFI and produce a 52 bp and 19 bp fragment. The mutant sequence does not cut, so it remains a 71 bp fragment.

Quantitative PCR to Assess Exon 9 in WG 1795

Exon 9 was examined in WG 1795 by quantitative PCR to assess whether both alleles were being amplified for the sequencing reaction. CEPH-1, WG 1756, WG 1758 and WG 1795 were all analyzed via quantitative PCR. The primers used were the same as those used to amplify the exon for sequencing. The exon 9 probe was labeled with FAM (Gibco) as the fluorescent dye and TAMRA (Gibco) as the quencher. The nucleotide sequence for the probe is:

5'-GCAAGTCCCTGCGCGCCTTC-3'

Quantitative PCR for exon 9 was performed using Qiagen protocols which contained HotStarTaq™ PCR buffer (Qiagen) and 200 µM dNTPs (Perkin Elmer). 1.0 U of HotstarTaq™ (Qiagen) was added to each reaction. The thermocycler used for quantitative PCR was the ABI 7700 (Perkin Elmer) with the following program:

- 1) 96°C for 15 minutes.
- 2) 96°C for 30 seconds.
- 3) Annealing temperature for 45 seconds.
- 4) 72°C for 1 minute.
- 5) Steps 2-4 repeated 35 times.
- 6) 72°C for 10 minutes.

As a control, a quantitative PCR reaction using an exon in the SCF-3 gene, used previously in an asthma study at the Montreal Genome Centre, with the primers and probe given in table # 3:

Table #3: Sequences of primers and probes used for SCF-3 in quantitative PCR

Primer/Probe	Sequence
SCF-3 Forward	tttgtgtgcttttctccaag
SCF-3 Reverse	tgagctactaaaatgaaactcagaaa
Probe	tcttccaaaagactacatgataaccctca

The probe was labeled with FAM (Gibco) as the fluorescent dye and TAMRA (Gibco) as the quencher. The quantitative PCR reaction used the PE Biosystems protocol. Each SCF-3 sample used 10X GeneAmp PCR Buffer (Perkin Elmer), 200 μ M dNTPs (Perkin Elmer), and 4.5 mM of $MgCl_2$ (Perkin Elmer). 1.0 U of Amplitaq Gold™ (Perkin Elmer) was added to each reaction. The ABI 7700 thermocycler (Perkin Elmer) was used with the same reaction conditions. Fluorescent levels were read and recorded at the end of step 4 of each cycle.

Chapter #5
Results

Sequencing Analysis of FTCD Patients

All 15 exons of the FTCD gene were sequenced from genomic DNA isolated from cell lines WG 1758, WG 1759 and WG 1795 as well as from CEPH-1 control DNA. Two heterozygous sequence changes were observed in WG 1758 and WG 1759, one in exon 4 and the other in exon 7. A single sequence change, apparently homozygous, was detected in exon 9 of WG 1795.

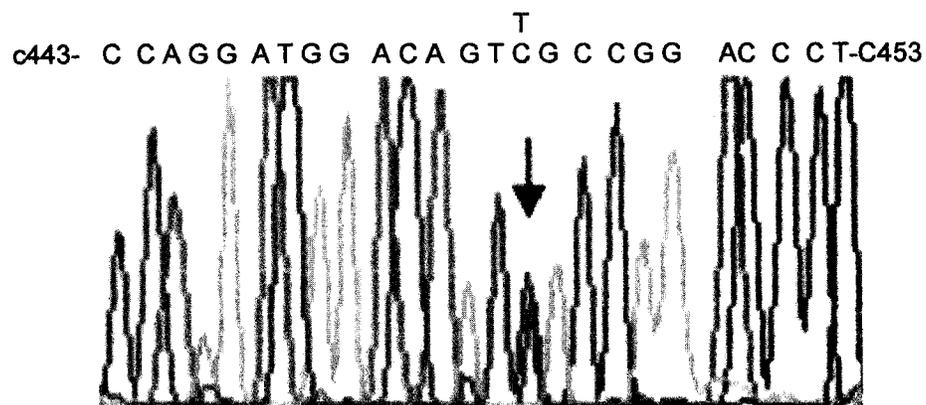
Cell lines WG 1758 and WG 1759 derived from siblings had the following two changes identified in FTCD. The first change is c457 C→T, which converts arginine to cysteine at amino acid position 135 (R135C). The sequencing analysis for c457 C→T is presented in figure #10. The second identified change is c940 G→C, which converts arginine to proline at amino acid position 299 (R299P). The sequencing analysis for c940 G→C is presented in figure #11.

Cell line WG 1795 has an insertion G at position c1033, and the sequencing analysis is presented in figure #12. As shown by the sequencing result, WG 1795 appears to be either homozygous or hemizygous for this insertion since no wild type sequence can be detected in this cell line. This frameshift causes an immediate stop codon right at the start of the linker region of the protein.

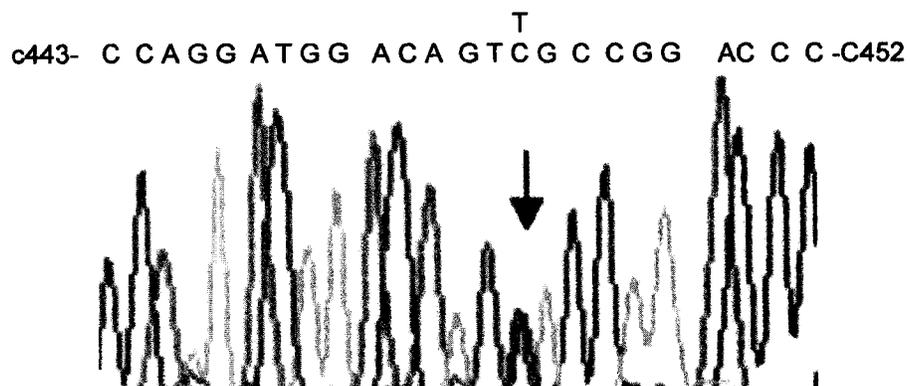
Population Analysis of the Identified Sequence Changes

Each of the sequence changes detected in the FTCD gene of patients was investigated in a panel of 100 control DNAs to verify that it was a mutation rather than a polymorphism present in the general population.

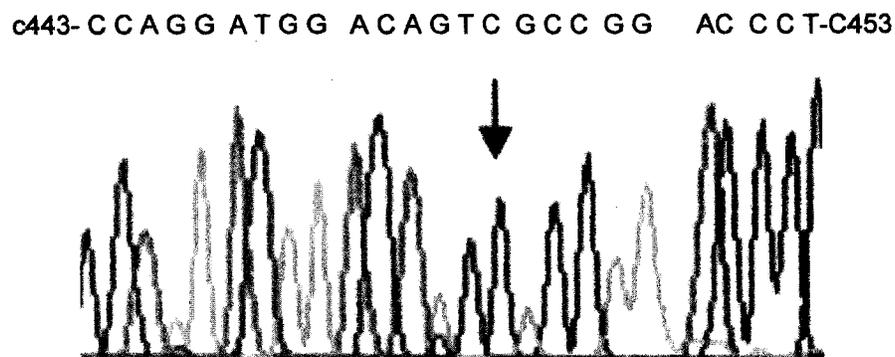
The c457 C→T sequence change results in the destruction of a *Hin*FI recognition site in exon 4 that is present in the wild type. PCR amplification of exon 4 results in a product of length 71 bp. Digestion of wild type DNA results in fragments of 52 bp and 19 bp in length. The PCR



WG 1758-EXON 4



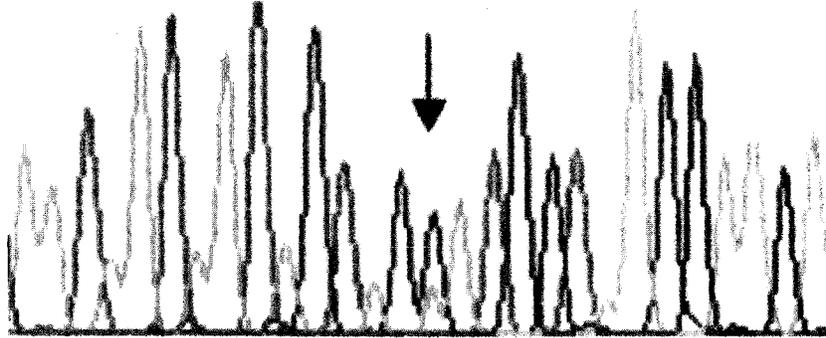
WG 1759-EXON 4



CEPH-1-EXON 4

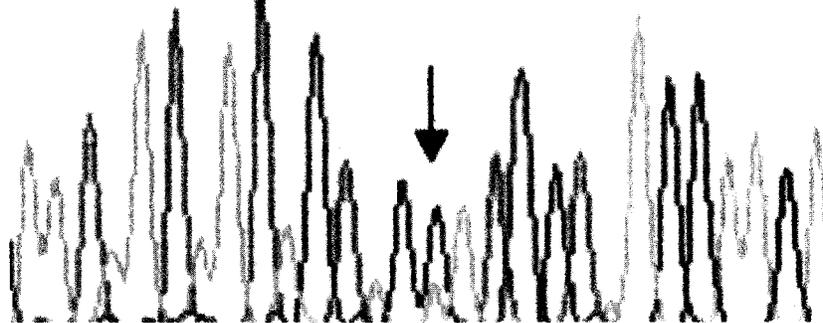
Figure #10: Sequencing analysis for c457 C→T. Sequencing from WG 1758, WG 1759 and CEPH-1 (wild type) are presented. The site of the mutation is indicated by the arrow. For the electropherograms, A is represented by green, C by blue, G by yellow, and T by red.

c926 -GG AGG AGGAG CAGC ^C GG AT CAG GC TGG T G - c953+ivs3



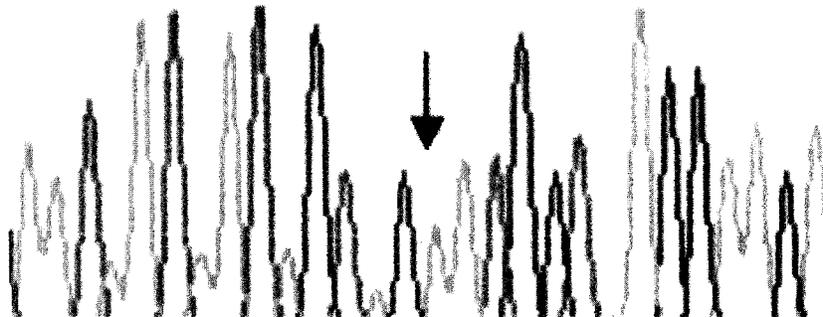
WG 1758-EXON 7

c926 -GG AGG AGG AGCAG C ^C GGAT CAG GC TGG T G - c953+ivs3



WG 1759-EXON 7

c926 -GG AGG AGG AGCAG C GGAT CAG GC TGG T G - c953+ivs3



CEPH-1 (WILD TYPE)-EXON 7

Figure #11: Sequencing analysis for c940 G→C. Sequences from WG 1758, WG 1759 and CEPH-1 (wild-type) are presented. The site of the mutation is indicated by the arrow. For the electropherograms, A is represented by green, C by blue, G by yellow and T by red.

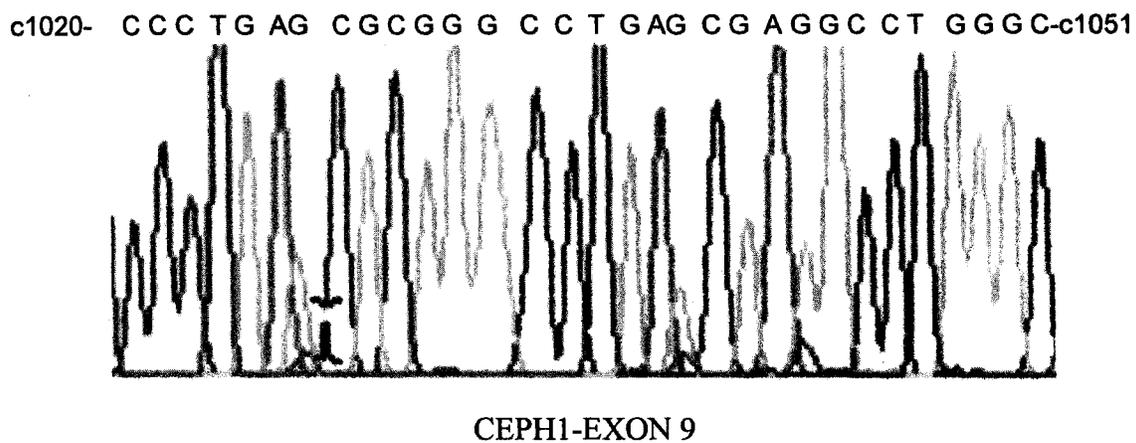
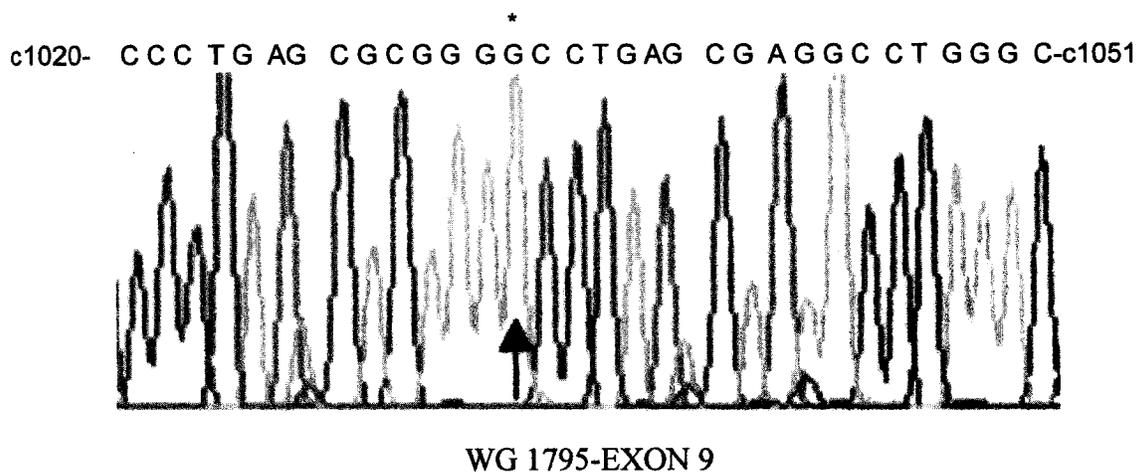


Figure #12: Sequencing analysis for c1033 insG. Sequences from WG 1795 and CEPH-1 (wild-type) are presented. The site of the insertion is indicated by the arrow. For the electropherograms, A is represented by green, C by blue, G by yellow and T by red.

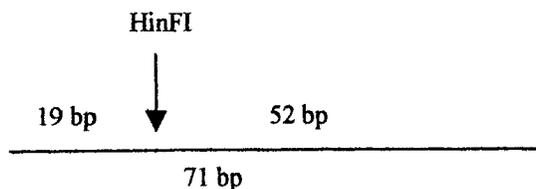
product is not cut when the c457 C→T change is present. Figure #13 shows the results of restriction endonuclease analysis of exon 4, which was visualized by electrophoresis on a 10% polyacrylamide gel (29:1 acrylamide/bis-acrylamide ratio). Uncut PCR product gave a single band of 71 bp. WG 1758 (heterozygous for c457 C→T) gave bands of 71 bp and 52 bp (the 19 bp fragment was not visualized in this system). Homozygous wild type DNA gave a single band of 52 bp. No mutant alleles were observed in the 100 normal individuals (200 chromosomes) assessed.

The c940 G→C sequence change results in the destruction of a AlwI recognition site in exon 7 that is present in the wild type. PCR amplification of exon 7 results in a product of length 603 bp. Digestion of wild type DNA results in fragments 340 bp and 263 bp in length. The PCR product is not cut when the c940 G→C change is present. Figure #14 shows the results of restriction endonuclease analysis of exon 7. Bands were visualized by electrophoresis on a 1.5% agarose gel. Uncut PCR product gave a single band of 603 bp in length. WG 1758 is heterozygous for c940 G→C gave bands 603 bp, 340 bp and 263 bp. Homozygotes for wild type DNA gave two bands 340 bp and 263 bp in length. . No mutant alleles were observed in the 100 normal individuals (200 chromosomes) assessed.

The c1033 insG sequence change results in the creation of an Eco0109I site in exon 9 that is present in the mutant sequence. PCR amplification of exon 9 results in a product 297 bp. Digestion of wild type DNA results in fragments 248 bp and 49 bp in length. Digestion of mutant DNA results in fragments 150 bp, 99 bp and 48 bp in length. Figure #15 shows the results of restriction endonuclease analysis of exon 9. Bands were resolved by electrophoresis on a 2% agarose gel. Uncut PCR product have a single band 297 bp. WG 1795 gave bands 150 bp and 99 bp in length (the 49 bp band cannot be visualized in this system). Homozygous wild

A

Wild Type:



Mutant:

Loss of HinFI
site

71 bp

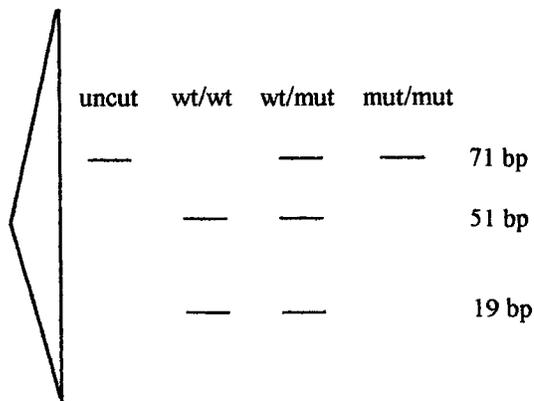
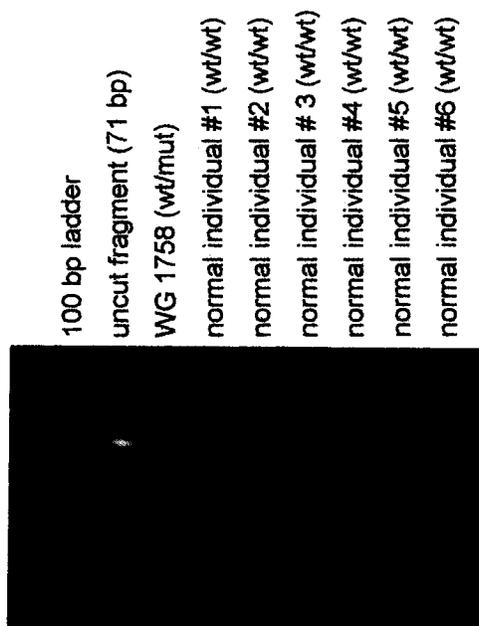
**B**

Figure #13: Example of c457 C→T restriction analysis. **A:** Diagram indicates the expected results of digestion with HinFI for wild type alleles and mutant alleles. **B:** Bands were visualized with a 10% acrylamide gel (19:1 ratio of acrylamide/bisacrylamide). Contents are indicated above each lane. All normal individuals tested were homozygous for the wild type allele.

A

Wild Type:

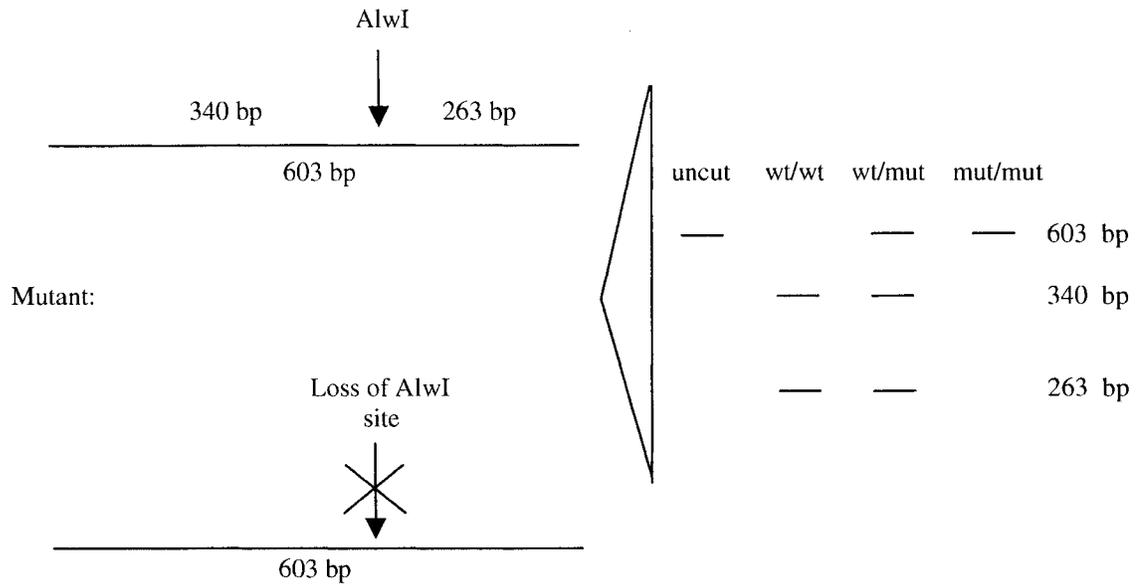
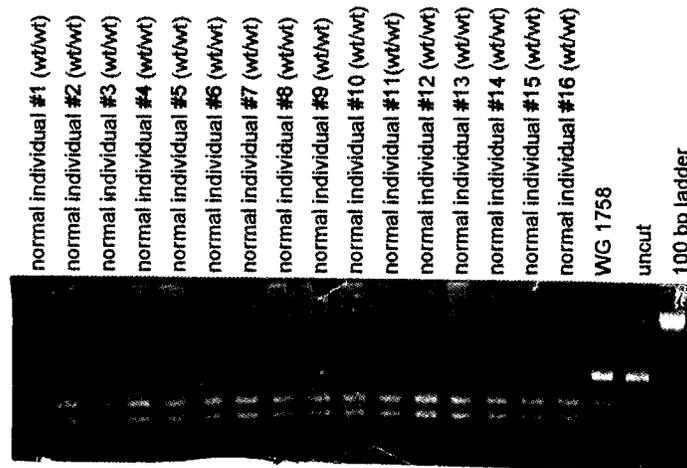
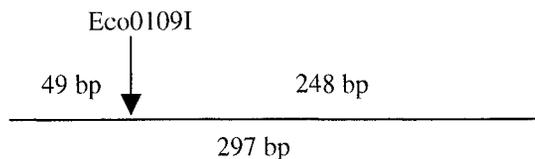
**B**

Figure #14: Example of c940 G→C restriction analysis. **A:** Diagram indicates the expected results of digestion with AlwI for wild type alleles and mutant alleles. **B:** Bands were visualized with a 1.5% agarose gel. Contents are indicated above each lane. All normal individuals tested were homozygous for the wild type allele.

A

Wild Type:



Mutant:

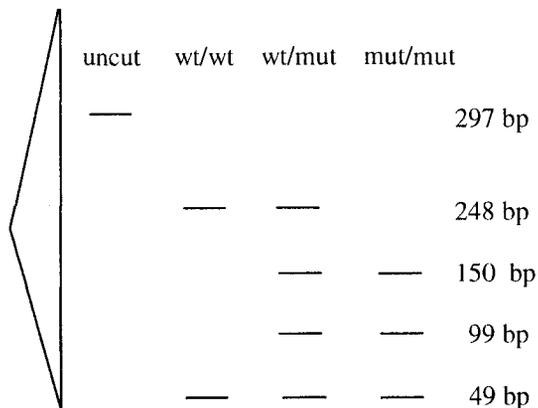
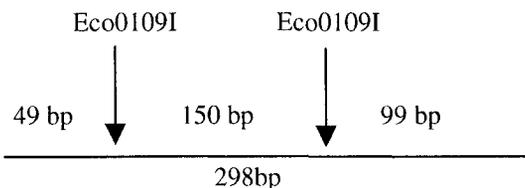
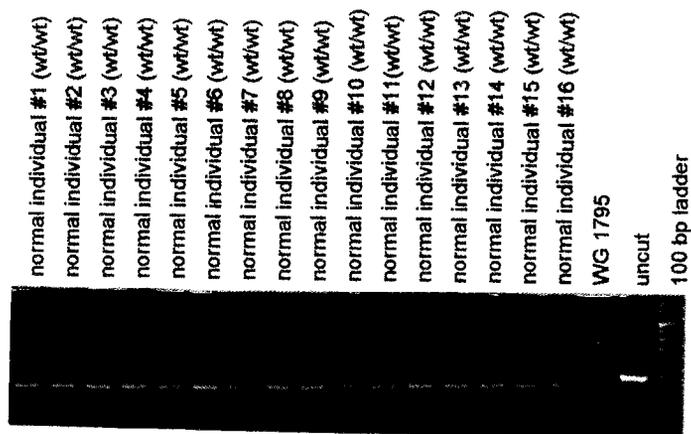
**B**

Figure #15: Example of c1033 insG restriction analysis. **A:** Diagram indicates the expected results of digestion with Eco0109I for wild type and mutant alleles. **B:** Bands were visualized with a 2.0% agarose gel. Contents are indicated above each lane. All normal individuals tested were homozygous for the wild type allele. WG 1795 appears to be homozygous or hemizygous for the c1033insG mutation.

type DNA gave a single band 248 bp in length. No mutant alleles were observed in the 100 normal individuals (200 chromosomes) assessed.

Quantitative PCR on WG 1795 to Determine Homozygosity or Hemizyosity

Quantitative PCR is a relatively new technique shown to be able to quantify the number of alleles present in a DNA sample. Real-time quantitative PCR has been used in experimental situations to detect gene amplifications in breast tumors with copy numbers reaching as high as 15. Results obtained from quantitative PCR in these studies were confirmed to be correct using Southern blotting techniques (Bieche et al., 1998). Quantitative PCR has also been used to detect deletions of tumor suppressors p15, p16, and p19 to provide predictive testing for individuals suffering from a genetic syndrome that involved cutaneous melanomas and nervous system tumors (Laurendeau et al., 1999). Quantitative PCR is also capable of differentiating between duplications and deletions at the same time, as it is used to differentiate between and provide predictive testing for Charcot-Marie-Tooth type 1A disease, which involves a duplication of peripheral myelin protein 22, and hereditary neuropathy with pressure palsies, which involves a deletion of the same gene (Aarskog and Vedeler, 2000).

Quantitative PCR evaluates the amount of PCR product at any given time interval by degrading a fluorescent probe using 5'-3' exonuclease activity of the Taq polymerase. To do this, a standard PCR is run; however, an oligonucleotide probe is included in the reaction that is capable of binding inside of the amplicon. The oligonucleotide probe contains a fluorescent dye and a quencher dye attached to the 5' and 3' ends respectively. As long as the probe remains intact, stimulation of the probe with light energy will not release any detectable fluorescence as the quencher absorbs the potential energy. If the probe binds to the region being amplified, the Taq polymerase will use its 5'-3' exonuclease activity to hydrolyze the probe as it moves

through. This releases the fluorescent dye from its quencher, making fluorescence detectable. Since a single fluorescent molecule is released for every PCR product molecule generated, it can be inferred that the amount of fluorescence measured in a sample is proportional to the amount of PCR product generated in a sample. This provides the means to directly measure the amount of PCR product generated.

Measuring fluorescence, it is possible to count the number of alleles in a given sample given the principles of PCR. Since the amount of PCR product is dependent on the amount of starting template, duplications or deletions will increase or decrease template copies respectively. Therefore, when comparing two samples with equal starting DNA concentrations and with one having a diploid copy number, if the other sample has lost an allele, there will be less fluorescence detected; if it has gained an allele, there will be more fluorescence detected. This is the theoretical basis for counting the number of alleles.

Two modifications are necessary due to technical complications. The first is due to reagent limitation. If two samples with different starting DNA concentrations are subjected to a PCR reaction with the standard 35-40 cycles and is quantified, there would be very little difference in the amount of fluorescence detected. This is because the PCR reagents become the limiting factor in the amplification process in the later cycles. Therefore, it is necessary to quantify product formation during the exponential phase of amplification, where the reagents are still in excess. This is why a threshold value is used. Instead of strictly measuring the final amount of fluorescence, the threshold value indicates after how many cycles a certain level of fluorescence is achieved; this level of fluorescence is set somewhere in the exponential phase of amplification. Therefore, when comparing two samples with equal starting DNA concentrations and with one having a diploid copy number, if the other sample has lost an allele, it will reach

the threshold at a later cycle; if it has gained an allele, it will reach the threshold at an earlier cycle.

The second modification is due to the technical impossibility of having exactly the same DNA concentration in each of the experimental samples. PCR is far more sensitive to differences in DNA concentration than can be accurately measured on a spectrophotometer. As a result, it is not possible to compare the threshold values between different samples. To overcome this, the threshold generated by the gene of interest is compared to the threshold generated by a control gene of known copy number from the same aliquot of DNA. The following calculation is then made for comparative purposes between different samples:

$$(\text{Threshold value for control}) - (\text{Threshold value for experimental gene})$$

If the experimental gene has the same copy number as the control, the above calculation should result in a value around 0. If there is a duplication in the experimental gene, then the above calculation should result in a positive number. If there is a deletion in the experimental gene, then the above calculation should result in a negative number.

A quantitative PCR approach was taken to assess whether WG 1795 was homozygous or hemizygous for c1033 insG in exon 9. An assessment of the results was made by taking the difference in the threshold value, which is defined as the PCR cycle where the amplicon has reached a set number of copies, between a control gene and the threshold value for exon 9 of FTCD. The control gene used in this experiment is Stem Cell Factor 3 (SCF-3), a gene whose function is not known to affect folate metabolism. Table #4 shows the results of this assessment.

Table #4: Quantitative PCR results for CEPH-1, WG 1756, WG 1758, and WG 1795. Threshold value is defined as which PCR cycle the amplicon reached a set number of copies as determined by fluorescence.

Sample	Threshold value for SCF-3	Threshold value for FTCD Exon 9	(Thres. SCF-3)- (Thres. FTCD exon 9)
CEPH-1 trial #1	25	25	0
CEPH-1 trial #2	25	25	0
CEPH-1 trial #3	26	26	0
WG 1756 trial #1	27	26	+1
WG 1756 trial #2	27	26	+1
WG 1758 trial #1	27	26	+1
WG 1758 trial #2	27	26	+1
WG 1795 trial #1	25	28	-3
WG 1795 trial #2	24	27	-3
WG 1795 trial #3	25	28	-3

Determination of the threshold value is illustrated by figure #16. The value 0 on the Y-axis represents the threshold level of fluorescence, so the X-axis value where each sample becomes positive for Y is the threshold value. Using this method, in figure #16 B which represents quantitative PCR on a CEPH-1 sample, the threshold value for FTCD and SCF-3 is 26. In figure #16 C which represents quantitative PCR on WG 1795, the threshold value for FTCD is 27 while for SCF-3 it is 24.

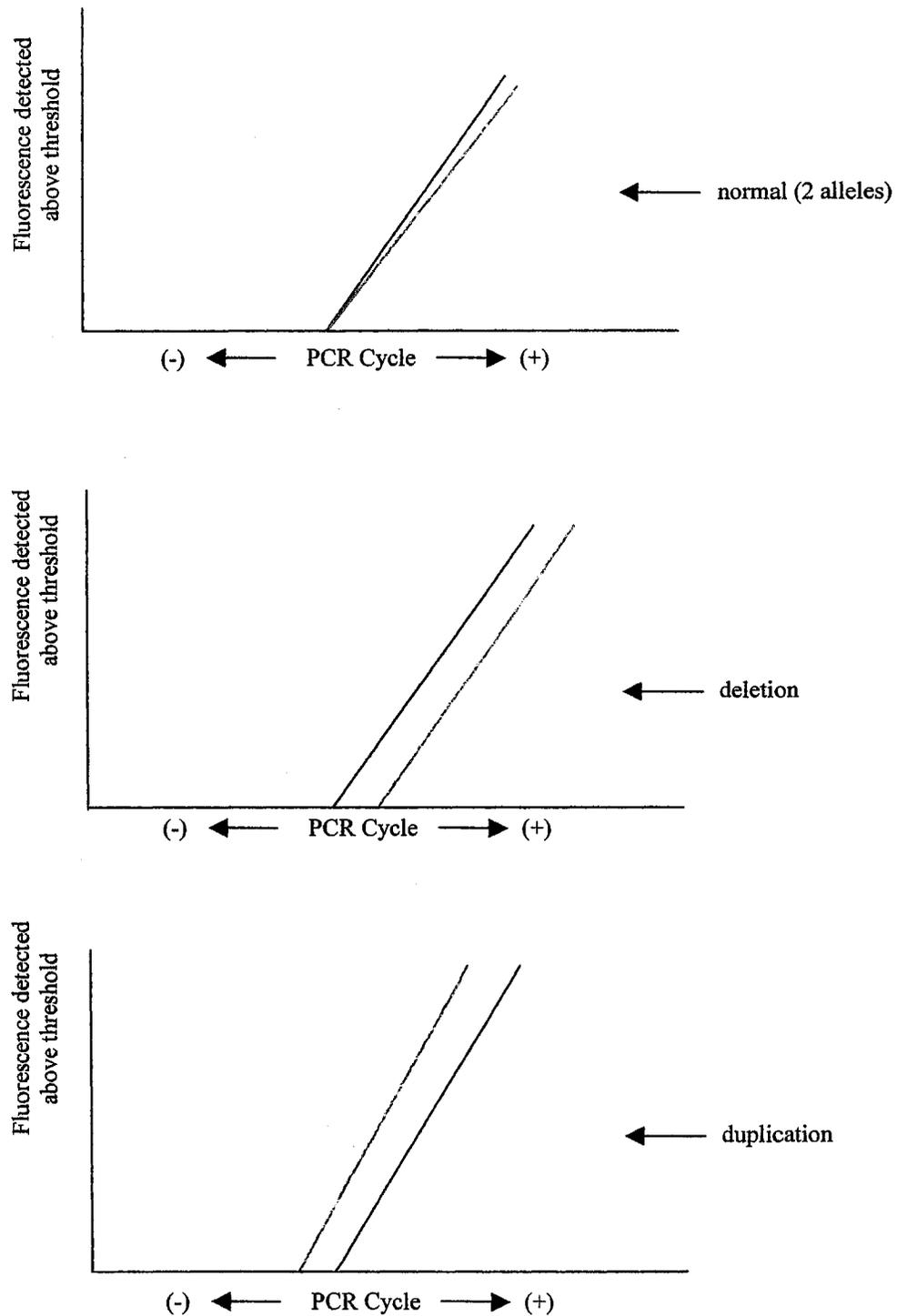


Figure #16 A: Theoretical quantitative PCR results expected for a normal situation, a deletion and a duplication. Control curve is given in red, while the experimental curve is given in green. Note that a deletion results in the experimental curve being shifted to the right relative to the control curve, while a duplication results in the experimental curve being shifted to the left relative to the control curve.

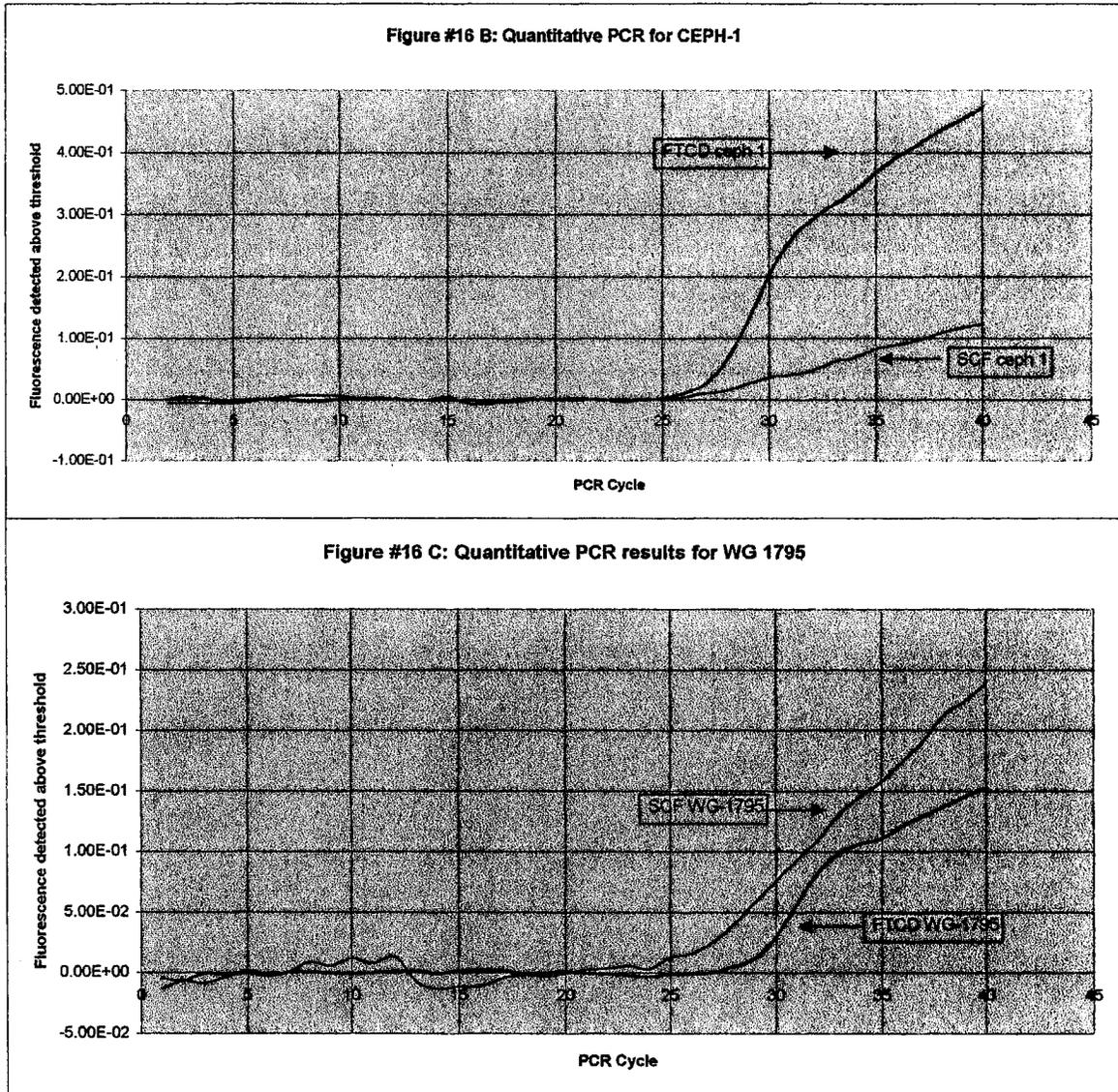


Figure #16B and C: Quantitative PCR for CEPH-1 and WG 1795. In B (performed on CEPH-1), notice that FTCD exon 9 and SCF-3 leave the x-axis at the same point. This is the result expected when FTCD exon 9 is present in two copies. In C (performed on WG 1795), notice that FTCD exon 9 leaves the x-axis at a later point than SCF-3. This is the result expected when one copy of FTCD exon 9 is deleted.

CEPH-1, WG 1756 and WG 1758 all have a normal copy number for exon 9 of FTCD. Note that in all cases that the difference between the threshold value for SCF-3 and FTCD exon 9 is between 0 and +1. These values indicate that in a sample where there is a normal copy number of exon 9, the difference in threshold values will always be 0 to +1. A noticeably different result was obtained for WG 1795, a sample which possibly has a deletion of exon 9 in FTCD. Although WG 1795 was subjected to exactly the same amplification conditions as CEPH-1, WG 1756, and WG 1758, the difference between the threshold value for SCF-3 and FTCD exon 9 is -3. Negative values indicate that the FTCD PCR reaction is not generating the same amount of product as the SCF-3 PCR reaction, and this can only be explained by a reduction in the amount of starting template for FTCD. This strongly suggests that WG 1795 has a deletion which minimally encompasses exon 9, as a deletion of one copy of exon 9 would reduce the amount of starting template which would affect the rate of amplification and the value determined for the threshold point.

Chapter #6
Discussion

Glutamate formiminotransferase deficiency has been proposed to be an inborn error of folate metabolism. Several apparently affected individuals have been identified but in most cases, it has not been possible to confirm the diagnosis. Identification of the human FTCD gene on chromosome 21 has made it possible to assess whether mutation of this gene is the molecular basis which underlies the disorder. We have cell lines from three patients with putative glutamate formiminotransferase deficiency, and sequencing of all 15 exons has allowed us to directly investigate this question.

WG 1758 and WG 1759

WG 1758 and WG 1759 are cell lines from two siblings diagnosed with glutamate formiminotransferase deficiency after massive levels of FIGLU was detected in one patient's urine. Direct sequencing of FTCD in these two cell lines identified two mutations that affect the function of glutamate formiminotransferase-cyclodeaminase. These two mutations were initially identified by direct sequencing and confirmed by restriction endonuclease digestion. The first mutation, c457 C→T, converts an arginine at amino acid position 135 to a cysteine (R135C). Homology comparison is shown in figure #17, and reveals that the arginine residue is highly conserved in all organisms sequenced (Solans et al., 2000) This amino acid change is not conservative as it changes a negatively charged residue to an uncharged one. The arginine residue is part of an extended loop in the formiminotransferase domain that interacts with the polyglutamate chain of the bound folate molecule. This loop extends from the surface of the formiminotransferase domain and into the cyclodeaminase domain (Kohls et al., 2000). Considering the importance of the polyglutamate chain in the binding and channeling of coenzyme between the formiminotransferase and cyclodeaminase domains, it is predicted that

H. sapiens
S. scrofa
M. musculus
G. gallus
S. pyogenes-ORF1
P. gingivalis-ORF1
T. acidophilus
T. maritima-TH0843

H. sapiens
S. scrofa
M. musculus
G. gallus
D. discoideum
S. pyogenes-ORF1
P. gingivalis-ORF1
T. acidophilus
T. maritima-TH0843

H. sapiens
S. scrofa
M. musculus
G. gallus
D. discoideum
S. pyogenes-ORF1
P. gingivalis-ORF1
T. maritima-TH0843
S. pyogenes-ORF2
P. gingivalis-ORF2
T. maritima-TH1560
C. acetobutylicum
C. tepidum
M. extorquens

H. sapiens
S. scrofa
M. musculus
G. gallus
D. discoideum
S. pyogenes-ORF2
P. gingivalis-ORF2
T. maritima-TH1560
C. acetobutylicum
C. tepidum
M. extorquens

H. sapiens
S. scrofa
M. musculus
G. gallus
D. discoideum
S. pyogenes-ORF2
P. gingivalis-ORF2
T. maritima-TH1560
C. acetobutylicum
C. tepidum
M. extorquens

Figure # 17: Primary sequence comparisons between organisms. The darker the shading, the more conserved the amino acid is between organisms. Arginine 135 is coloured purple while arginine 299 is coloured red. The site of the stop codon generated by c1033 insG is indicated in green. This is in the middle of the linker region that connects the formiminotransferase domain with the cyclodeaminase domain (Solans et al., 2000)

the loss of the conserved arginine in this loop may either affect the binding of folate to the octameric enzyme or affect the efficiency of channeling between the two functional domains.

Another possibility is that the mutation affects mRNA stability. Sequencing of cDNAs from fibroblast cultures of WG 1758 and WG 1759 has been performed previously (Estivil and De la Luna, unpublished results). Their research efforts failed to identify c457 C→T although readable sequences were obtained for this region of the cDNA. This suggests that the cDNA containing the sequence change was not present, perhaps because the mRNA was unstable or rapidly degraded, thus not available to act as a template for cDNA synthesis.

The second change observed in WG 1758 and WG 1759 is c940 G→C, which converts an arginine at amino acid position 299 to a proline. As shown by figure #17, the arginine is only partially conserved based on homology comparisons. Residues at this position are positively charged, as indicated by the presence of either arginine or lysine (Solans et al., 2000). Residues 288-316 in the formiminotransferase domain form a C-terminal domain α -helix that makes extensive hydrogen bonding contacts within the formiminotransferase dimeric interface (Kohls et al., 2000). The introduction of a proline at this position is significant in two possible ways. One possibility is a weakening of the hydrogen bonding between dimerizing domains as proline is not capable of hydrogen bonding while arginine is. The other possibility is that the introduction of a proline at position 299 disrupts the α -helix, preventing any dimerization of the formiminotransferase domains. This possibility will result in a non-functional enzyme, as dimerization is absolutely essential for function. Earlier attempts to identify sequence changes in WG 1758 and WG 1759 in cDNA were unsuccessful in identifying c940 G→C because no readable sequences could be obtained for this segment of the FTCD gene (Estivill and De la Luna, unpublished results).

Neither sequence change was observed in 100 normal individuals analyzed which supports the notion that these changes are responsible for glutamate formiminotransferase deficiency in WG 1758 and WG 1759. Since a polymorphism is defined by a frequency of 1%, it should have been observed at least twice in the 200 alleles assessed (Beaudet et al., 2001). With the changes not being polymorphic, it is much more likely that the changes are disease causing.

The identification of glutamate formiminotransferase deficiency in the patient represented by WG 1758 resulted in all family members being assessed for their biochemical characteristics for this disease. Neither of the parents show any form of the disease phenotype. Both were in good physical and mental health and were not found to excrete any FIGLU (Perry et al., 1975). This suggests that one functional copy of the gene is more than sufficient to meet the histidine degradation demands of the human body.

WG 1795

Direct sequencing of the FTCD gene in WG 1795 indicates that there is an insertion of a G at position c1033. The insertion changes the reading frame and creates a stop codon two positions downstream in the middle of linker region between the formiminotransferase domain and the cyclodeaminase domain. Previous work has shown that the formiminotransferase domain is functional when expressed without the cyclodeaminase domain as long as it can self-dimerize (Murley and Mackenzie, 1995). As a result of this insertion, WG 1795 will produce a transferase fragment capable of self-dimerizing and catalyzing the formation of 5-formiminotetrahydrofolate. No functional cyclodeaminase will be synthesized as a result of this mutation due to the stop codon in the linker region.

No normal allele was detected in this individual, suggesting that either the patient was homozygous for this sequence change, or that one copy of FTCD gene in the patient had been lost due to deletion, resulting in hemizyosity. Results from quantitative PCR also strongly suggests that the mutation is in the hemizygous state. Quantitative PCR in this experiment used genomic DNA from CEPH-1, WG 1756, and WG 1758 in the analysis because sequencing analysis of these samples indicated the presence of two intact alleles of FTCD. In the situation of CEPH-1, the difference between the SCF-3, which served as the control gene, and exon 9 of FTCD was 0. In WG 1756 and WG 1758, this difference was +1. These observations establish that the threshold difference between SCF-3 and FTCD exon 9 in the wild type situation has a range of 0 and +1. A strikingly different observation is seen for WG 1795. Unlike CEPH-1, WG 1756 and WG 1758, WG 1795 has a threshold difference of -3. A negative value indicates that amount of PCR product for FTCD exon 9 is much less than SCF-3. Since WG 1795 was subjected to the same amplification conditions as the other samples, these results support the suggestion that WG 1795 is hemizygous for c1033 insG, with the other copy of exon 9 eliminated by a deletion.

Consistency of Rowe's Hypothesis With Patient Phenotypes

There is phenotypic variation among patients with FTCD deficiency. Patients presenting with the mild form of the disease, marked by slight mental and developmental retardation and hypotonia, excrete very high levels of FIGLU in the urine. Patients presenting with the severe form of the disease, marked by severe mental retardation and hematological abnormalities, excrete much lower levels of FIGLU. Based on the FIGLU excretion levels, it was proposed that patients with the mild form of the disease had a block in the formiminotransferase reaction, while patients with the severe form of the disease had blocks in the cyclodeaminase reaction. Blocks

in the formiminotransferase reaction would result in all FIGLU generated being excreted in the urine, while blocks in the cyclodeaminase will have some of the generated FIGLU being converted to 5-formiminotetrahydrofolate (Rowe, 1983). To date, it has not been possible to test this hypothesis.

Based on the genetic composition of WG 1795, there is an opportunity to test the validity of this hypothesis. The c1033 insG mutation produces functional transferase but no functional cyclodeaminase in one allele. Because no wild-type allele is present, no functional cyclodeaminase is produced by the other allele. According the above hypothesis, this patient would present with the severe form of the disease and excrete lower levels of FIGLU; however, this is not the case. Levels of FIGLU excretion from WG 1758, WG 1759, WG 1795 compared to the severely affected Japanese patients is given in table #5:

Table #5: Urinary FIGLU excretion levels for WG 1758, WG 1759, WG 1795 and the severely affected patients from Japan (Erbe, 1986). (nr=not reported)

Patient	Urinary FIGLU Excreted on Normal Diet ($\mu\text{mol}/24\text{ h}$)	Urinary FIGLU Excreted After Histidine Loading ($\mu\text{mol}/24\text{ h}$)
WG 1758	3897	nr
WG 1759	2833	nr
WG 1795	1700	nr
CK (Japanese Patient Case #1)	nr	40-200
HK (Japanese Patient Case #2)	768	1939
YA (Japanese Patient Case #3)	nr	258-6279
YN (Japanese Patient Case #4, sibling of YA)	nr	205-262
SK (Japanese Patient Case #5)	nr	nr

Patients represented by WG 1758 and WG 1759 have blocks in the formiminotransferase reaction, while the patient represented by WG 1795 has a block in cyclodeaminase reaction. As can be seen by table #7, blocks in the formiminotransferase and cyclodeaminase both result in high levels of FIGLU excretion in the absence of histidine loading. Clinically, it also appears as though blocks in the formiminotransferase or in the cyclodeaminase results in the same phenotype. All three patients suffer from slight mental retardation as shown by delayed speech

development and hypotonia (Beck et al., 1981). Although the stability of the mRNA produced by c1033 insG has yet to be assessed, these observations suggest that Rowe's hypothesis is not true, and that the cause of the severe and mild forms of the disease is due to another factor.

Summary and Conclusions

In summary, genomic DNA extracted from cell lines derived from three patients with suspected glutamate formiminotransferase deficiency were analyzed through PCR and sequencing of individual exons. Cell lines WG 1758 and WG 1759 represent two siblings of Germanic descent who were found to excrete very large amounts of FIGLU after coming to medical attention due to learning disabilities. Both siblings are heterozygous for c457 C→T and c940 G→C. The c457 C→T changes a conserved arginine to a cysteine in a loop involved in the binding of formiminotetrahydrofolate to the enzyme. The c940 G→C mutation converts an arginine to a proline in an α -helix essential for the dimerization of the formiminotransferase domain. Cell line WG 1795 represents a patient of Danish descent who was found to have slight hypersegmentation of leukocytes and slight mental retardation in the presence of FIGLU excretion. In addition, the patient suffered from recurring otitis media and severe pulmonary infections. The patient appears to be hemizygous for c1033 insG with quantitative PCR indicating the presence of a deletion which minimally encompasses exon 9 of FTCD. All three changes were confirmed to be mutations by their absence in 100 normal individuals. These findings represent the first proof that mutations in the gene encoding FTCD underlies this clinical condition.

Glutamate formiminotransferase deficiency is a clinically heterogeneous disease. Some patients present with a severe phenotype, which is clinically marked by mental retardation, hematological abnormalities and elevated serum folate levels. Other patients present with a mild phenotype, which is clinically marked by delayed speech development and general hypotonia. Biochemically, the two phenotypes can be differentiated by measuring FIGLU levels in the urine. In general, patients with the severe phenotype have elevated FIGLU levels in the urine in

response to histidine loading, while patients with the mild phenotype have elevated FIGLU levels in the urine while consuming a normal diet. In addition, the levels of FIGLU in the urine for patients with the mild phenotype is substantially higher than what is observed in patients with the severe phenotype. Because of these observations, it was hypothesized that the mild phenotype was a result of blocks in the formiminotransferase domain, while the severe phenotype was a result of blocks in the cyclodeaminase domain (Rowe, 1983). The patient represented by WG 1795 presents with the mild phenotype even though the patient contains a metabolic block at the cyclodeaminase reaction. As a result, it is very likely that the above hypothesis is incorrect.

Claims to Originality

The following are original contributions to the knowledge of glutamate formiminotransferase deficiency, an inborn error of folate metabolism:

- 1) Identification of the first mutations responsible for the deficiency.
- 2) Evidence that suggests that the severe form of the disorder is not due to blocks in the cyclodeaminase reaction as originally hypothesized by Rowe in 1983.

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