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**Characterization of a Gene Encoding the Human Mitochondrial
C₁-Tetrahydrofolate Synthase and Submitochondrial
Localization of the Protein**

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C₁-Tetrahydrofolate Synthase and Submitochondrial
Localization of the Protein**

by

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Dedication

To my beloved husband, Manoj

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**Characterization of a Gene Encoding the Human Mitochondrial
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Localization of the Protein**

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C₁-tetrahydrofolate (THF) synthase is a eukaryotic trifunctional enzyme that possesses the activities 5,10-methylene-THF dehydrogenase, 5,10-methenyl-THF cyclohydrolase, and 10-formyl-THF synthetase. These activities catalyze three sequential folate-mediated one-carbon interconversions resulting in the synthesis of formate. In the yeast, *Saccharomyces cerevisiae*, C₁-THF synthase is present in the cytoplasm as well as in the mitochondria. A mammalian cytoplasmic isozyme of C₁-THF synthase has been characterized for several years, but the mitochondrial isozyme in mammals had not been previously identified. This dissertation describes the cDNA cloning and characterization of a human mitochondrial C₁-THF synthase.

Human chromosome 6 carries the gene encoding the mitochondrial isozyme, which spans 236 kilobase pairs. The protein encoded by the full-length cDNA (978 amino acids) is 61% identical to the cytoplasmic isozyme and has an N-terminal mitochondrial targeting sequence. Expression of the full-length cDNA (2934 bp) clone in Chinese Hamster Ovary (CHO) cells showed that the protein is localized in the mitochondria.

The gene encoding the mitochondrial C₁-THF synthase isozyme is expressed in most of the human tissues examined and is high in placenta, followed by thymus and spleen. The transcript is distinct from that of the cytoplasmic isozyme in size and tissue distribution. The transcriptional start site of the gene was mapped as well. A short transcript representing the 5'-end of the gene was also observed by Northern blot analysis and was shown to arise from alternative splicing. The possibility of the short transcript encoding a functional protein was investigated. A cDNA of the short transcript of C₁-THF synthase was cloned into an *E. coli* expression vector and the recombinant protein (short isoform) was purified. Although catalytically inactive, the purified short isoform was successfully used to make polyclonal antibodies specific for mitochondrial C₁-THF synthase.

Earlier studies done in our lab had also shown that adult rat liver mitochondria can metabolize carbon 3 of serine into formate by a folate-dependent pathway. To confirm that this one-carbon metabolism is occurring inside the mitochondria, we further explored the sublocalization of human mitochondrial C₁-THF synthase.

Analysis of mitochondrial subfractions from CHO cells transfected with the full-length cDNA clone demonstrated that the human mitochondrial C₁-THF synthase was associated with the matrix side of the inner mitochondrial membrane. Subfractionation of rat spleen mitochondria showed that mitochondrial C₁-THF synthase was localized in the inner membrane in adult mammalian tissues as well. During the course of this work, studies by other members of the lab revealed that the recombinant human mitochondrial C₁-THF synthase is monofunctional, possessing only the 10-formyl-THF synthetase activity. Since the human mitochondrial isozyme appears to be an inner membrane protein and also monofunctional, we hypothesized that it might interact with an unidentified enzyme that could provide the missing 5,10-methylene-THF dehydrogenase and 5,10-methenyl-THF cyclohydrolase activities. Initial attempts to identify such an interaction were unsuccessful. In summary, we have identified the gene encoding the human mitochondrial C₁-THF synthase, cloned and characterized it as well. The protein encoded by this gene was shown to be localized in the mitochondria, specifically in the inner mitochondrial membrane in a mammalian cell line.

Table of Contents

List of Tables.....	x
List of Figures	xi
CHAPTER 1 INTRODUCTION	1
1.1 The Biochemistry of Folates	3
1.2 Folate-mediated One-Carbon Metabolism.....	5
1.3 Compartmentation of Folate-mediated One-Carbon Metabolism.....	9
1.4 Role of Mitochondrial One-carbon Metabolism in Cytoplasmic Processes	13
1.5 Importance of Mammalian Mitochondrial C ₁ -THF synthase	18
1.6 The Domain Structure of C ₁ -THF Synthase	19
1.7 Research History of Mammalian Cytoplasmic and Mitochondrial C ₁ -THF Synthase.....	22
1.8 Current Study	24
CHAPTER 2 cDNA Cloning and Immunolocalization of Human Mitochondrial C₁-THF Synthase in Chinese Hamster Ovary (CHO) Cells	26
2.1 OVERVIEW.....	26
2.2 EXPERIMENTAL PROCEDURES	27
2.2.1 cDNA Identification.....	27
2.2.2 PCR Amplification of 5'-exon (Exon 1).....	29
2.2.3 PCR Amplification of Partial cDNA.....	29
2.2.4 SOE-PCR to generate Full-length Human Mitochondrial C ₁ -THF Synthase cDNA	30
2.2.5 TOPO Cloning of Full-length cDNA.....	31
2.2.6 Site Directed Mutagenesis.....	34
2.2.7 Subcloning of pcDNA3.1-humito into pBluescript II KS (+/-)	35
2.2.8 PCR Amplification of pBluescript II KS (+/-)/pcDNA3.1-humito.....	36

2.2.9	Subcloning of Mutated cDNA into pcDNA3.1-humito Larger Fragment.....	37
2.2.10	CHO Cell Transfection.....	38
2.2.11	Preparation of Cell Homogenates and Subcellular Fractions.....	39
2.2.12	Immunoblotting.....	41
2.3	RESULTS.....	41
2.3.1	cDNA Identification.....	41
2.3.2	PCR amplification of Exon 1 and Partial cDNA.....	45
2.3.3	SOE-PCR & TOPO Cloning.....	45
2.3.4	Gene Structure.....	46
2.3.5	CHO Cell Expression and Subcellular Localization.....	50
2.4	DISCUSSION	53

CHAPTER 3	Tissue Distribution of the mRNA and Transcript Mapping of the 5'-end	56
3.1	OVERVIEW.....	56
3.2	EXPERIMENTAL PROCEDURES	57
3.2.1	Northern Analysis	57
3.2.2	5' RNA Ligase Mediated Rapid Amplification of cDNA Ends (5' RLM-RACE)	60
3.3	RESULTS.....	63
3.3.1	Northern Analysis	63
3.3.2	Transcript Mapping.....	65
3.4	DISCUSSION	68

CHAPTER 4 Mapping of the 3'-end of the Short Transcript and cDNA Cloning, Expression, and Purification of the Short Isoform of Human Mitochondrial C₁-THF Synthase	70
4.1 OVERVIEW	70
4.2 EXPERIMENTAL PROCEDURES	71
4.2.1 3' RNA Ligase Mediated Rapid Amplification of cDNA Ends (3' RLM-RACE)	71
4.2.2 cDNA Cloning of Short Isoform of Mitochondrial C ₁ -THF Synthase	72
4.2.3 Expression and Purification of Short Isoform	74
4.2.4 Expression and Purification of TEV protease	77
4.2.5 Polyclonal Antibody Production	79
4.2.6 Enzyme Assay of Short Isoform	80
4.3 RESULTS	81
4.3.1 Alternative Splicing	81
4.3.2 cDNA Cloning, Expression, and Purification of Short Isoform	85
4.3.3 Specificity of Anti-mito-C ₁ -THF Synthase Antibodies	88
4.4 DISCUSSION	92
CHAPTER 5 Sublocalization of Human Mitochondrial C₁-THF Synthase	97
5.1 OVERVIEW	97
5.2 EXPERIMENTAL PROCEDURES	98
5.2.1 Mitochondrial Isolation and Submitochondrial fractionation from CHO/pcDNA3.1-humito cells	98
5.2.2 Immunoblotting of Submitochondrial Fractions	100
5.2.3 Alkaline Carbonate Extraction of CHO/pcDNA3.1-humito Mitochondria	101
5.2.4 Protease Treatment of CHO/pcDNA3.1-humito Mitochondria	102

5.2.5	Mitochondrial Isolation and Submitochondrial fractionation from Rat Spleen	103
5.2.6	Isolation of Mitochondria from Human Placenta.....	104
5.2.7	Immunoprecipitation of Human Mitochondrial C ₁ -THF Synthase...	105
5.3	RESULTS.....	105
5.3.1	CHO Cell Expression and Submitochondrial Localization	105
5.3.2	Alkaline Carbonate Extraction of CHO/pcDNA3.1-humito Mitochondria	108
5.3.3	Membrane Topology of Human Mitochondrial C ₁ -THF Synthase...	110
5.3.4	Submitochondrial Localization of Rat Spleen Mitochondrial C ₁ -THF Synthase.....	113
5.3.5	Expression Level of Mitochondrial C ₁ -THF Synthase in Tissues and CHO Cells	115
5.3.6	Immunoprecipitation of Human Mitochondrial C ₁ -THF Synthase...	116
5.4	DISCUSSION	119
CHAPTER 6 CONCLUDING REMARKS		125
6.1	Dissertation Summary	125
6.2	Conclusions and Future Work.....	128
APPENDIX I Abbreviations.....		132
APPENDIX II Materials.....		135
APPENDIX III Bacterial Strains and Plasmids.....		138
	Bacterial Strains	138
	Plasmids	139
APPENDIX IV Protocols.....		141
BIBLIOGRAPHY		165
VITA.....		180

List of Tables

Table 2.1	Intron/exon Splice Junctions of the Human Mitochondrial C ₁ -THF Synthase Gene.....	49
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List of Figures

Figure 1.1	Structure of Tetrahydrofolate.	5
Figure 1.2	Major Pathways of Folate-dependent One-carbon Metabolism.....	8
Figure 1.3	Compartmentation of One-carbon Metabolism in <i>Saccharomyces cerevisiae</i>	12
Figure 1.4	Proposed Mammalian One-carbon Cycle.	16
Figure 1.5	Domain Structure of C ₁ -THF Synthase.....	21
Figure 2.1	SOE-PCR to create Full-length Human Mitochondrial C ₁ -THF synthase cDNA.....	31
Figure 2.2	TOPO Cloning of Full-length cDNA into pcDNA3.1D/V5-His-TOPO Vector.	34
Figure 2.3	Schematic Representation of pcDNA3.1-humito.....	38
Figure 2.4	Alignment of Human and Mouse Mitochondrial C ₁ -THF Synthases with Human Cytoplasmic C ₁ -THF Synthase.	44
Figure 2.5	Agarose Gel of Full-length cDNA generated by SOE-PCR.	46
Figure 2.6	Intron/exon Structure of the Human Mitochondrial C ₁ -THF Synthase Gene.	48
Figure 2.7	Subcellular Localization of Epitope-tagged Human Mitochondrial C ₁ -THF Synthase Expressed in CHO cells.	52
Figure 3.1	Northern Blot Analysis of Mitochondrial C ₁ -THF Synthase Transcripts in Adult Human Tissues.....	65
Figure 3.2	5'-Flanking Sequence and Exon 1 of the Human Mitochondrial C ₁ -THF Synthase Gene.	67
Figure 4.1	Schematic Representation of pMal Expression System for	

	Short Isoform Purification.....	77
Figure 4.2	Purification of TEV Protease.	79
Figure 4.3	Agarose gel of 3' RACE "inner" PCR products.	82
Figure 4.4	Alternative Splicing of the Human Mitochondrial C ₁ -THF Synthase Transcript.	83
Figure 4.5	Variable 3'-Splice Site Selection at the Exon 6/7 junction.	85
Figure 4.6	Purification of the Short Isoform of Human Mitochondrial C ₁ -THF Synthase.....	88
Figure 4.7	Specificity of Anti-mito-C ₁ -THF Synthase Antibodies.	91
Figure 4.8	Alignment and Structure Comparison of the Short Isoform of Human Mitochondrial C ₁ -THF Synthase (<i>hmito</i>) and Human Cytoplasmic Dehydrogenase/Cyclohydrolase (D/C) Domain (<i>hcyto</i>).	96
Figure 5.1	Submitochondrial Localization of Epitope-tagged Human Mitochondrial C ₁ -THF Synthase Expressed in CHO Cells.	107
Figure 5.2	Alkaline Carbonate Extraction of CHO/pcDNA3.1-humito Mitochondria.	109
Figure 5.3	Membrane Topology of Human Mitochondrial C ₁ -THF Synthase Expressed in CHO Cells.....	112
Figure 5.4	Submitochondrial Localization of Endogenous Rat Spleen Mitochondrial C ₁ -THF Synthase.....	114
Figure 5.5	Expression Level of Mitochondrial C ₁ -THF Synthase.	116
Figure 5.6	Immunoprecipitation of Human Mitochondrial C ₁ -THF Synthase...	118
Figure 6.1	Proposed Model for THF-mediated One-Carbon Metabolism in Adult Mammalian Mitochondria.....	127

CHAPTER 1

INTRODUCTION

Folates are essential for most forms of life on earth to facilitate one-carbon transfers. They are key to several major cellular processes including *de novo* purine and thymidylate synthesis, methyl group biogenesis, serine and glycine interconversion, vitamin metabolism, and mitochondrial and chloroplast protein biosynthesis (Blakley, 1969). An exception to this are *archaea*, which use modified folates (one example being methanopterin) for one-carbon transfers (Van Beelen *et al.*, 1984). Most bacteria, plants and lower eukaryotes have the ability to synthesize folates to meet their requirement for one-carbon transfers (Blakley, 1968). Higher eukaryotes depend on folate intake through diet or on intestinal microorganisms to satisfy their needs (Cooper, 1985). Since the discovery of folic acid, folate-mediated one-carbon metabolism has drawn the attention of many researchers. It gained more interest when the clinical manifestations of folate deficiency became evident in the form of megaloblastic anemia. Also, there are several recent reports showing that folate deficiency in pregnant women is associated with neural tube defects (NTDs) in infants (Barber, 1999). Folic acid supplementation in pregnant women's diet was shown to bring down the risk of NTDs by up to 70%, thereby turning the focus onto the genes encoding the numerous proteins of folate-dependent one-carbon metabolism and of folate transport (Finnell, 2003). It has been reported that mouse

embryos lacking a folate transport protein exhibit severe defects in embryonic development, including neural tube defects and premature death (Piedrahita, 1999). Premature death in these embryos could be prevented by folate supplementation in the form of folinic acid, which affected the expression of several genes including those for cell proliferation and growth factors (Spiegelstein, 2004). Also, there are studies showing the effect of folate deficiency on blood homocysteine levels, which when elevated (hyperhomocysteinemia) has been associated with vascular disorders (Finkelstein, 2000). Since folates are crucial for nucleotide biosynthesis, folate-dependent enzymes are also targets of drug therapy. Methotrexate, trimethoprim, and 5-fluorouracil are some of the drugs that have had remarkable success in chemotherapy. In our lab, several key aspects of folate metabolism have been studied for years using the yeast, *Saccharomyces cerevisiae*, as a model organism. Recently, based on our observations in yeast, research is being done to gain a better understanding of one-carbon metabolism in mammals as well.

This introduction will cover the biochemistry of folates, an overview of folate-mediated one-carbon metabolism and in particular, the research that led us to further explore one of the folate-dependent enzymes, C₁-tetrahydrofolate (THF) synthase, a trifunctional enzyme that converts 5,10-methylene tetrahydrofolate into formate. This dissertation will focus on the cDNA cloning and characterization of the human mitochondrial C₁-THF synthase, a central player in one-carbon metabolism.

1.1 The Biochemistry of Folates

The discovery of folic acid resulted from research conducted by several laboratories with different biological systems including chicken, primates, alfalfa, and lactic acid bacteria (Blakley, 1969). In 1940, it was reported that an unidentified factor facilitates the growth of *Lactobacillus casei* (Snell and Peterson, 1940). A year later, folic acid was isolated from spinach leaves in purified form (Mitchell *et al.*, 1941), and hence the name (Latin: folium=leaf). Its basic chemical structure was later postulated to be N-[4-{(2-amino-4-hydroxy-6-pteridiny)methyl}amino}benzoyl] glutamic acid (Angier *et al.*, 1946) and they suggested the name “pteroylglutamic acid”. The synonym “folic acid” given by Mitchell *et al* is still preferred. Folic acid consists of a pteridine ring attached to a *p*-aminobenzoic acid moiety by a C⁹-N¹⁰ bond (together called pteronic acid), which is linked *via* an amide bond through its carboxyl moiety to the amino group of L-glutamic acid (see Figure 1.1).

One carbon transfers are mediated by tetrahydrofolate (THF), the biologically active form of folic acid that is reduced at positions N⁵, C⁶, C⁷, and N⁸ of the pteridine ring (Figure 1.1). THF carries activated one carbon units at positions N⁵ and/or N¹⁰ to produce 5-methyl, 5-formyl, 5,10-methylene, 5,10-methenyl, and 10-formyl derivatives (Blakley, 1969; Cossins, 1984; MacKenzie, 1984).

Naturally occurring folates occur as poly- γ -glutamate conjugates with 5 to 8 glutamate residues, depending on the source (Cossins, 1984). Intracellularly, the

major forms of folate found are pteroylpolyglutamates and polyglutamylation is catalyzed by a single enzyme (folypolyglutamate synthetase) in most organisms (McGuire and Coward, 1984). Polyglutamate tails serve to retain folates within the cell, to increase its affinity for folate-dependent enzymes, and in substrate channeling of folates (MacKenzie, 1984; Schirch and Strong., 1989; Shane, 1989; Appling, 1991).

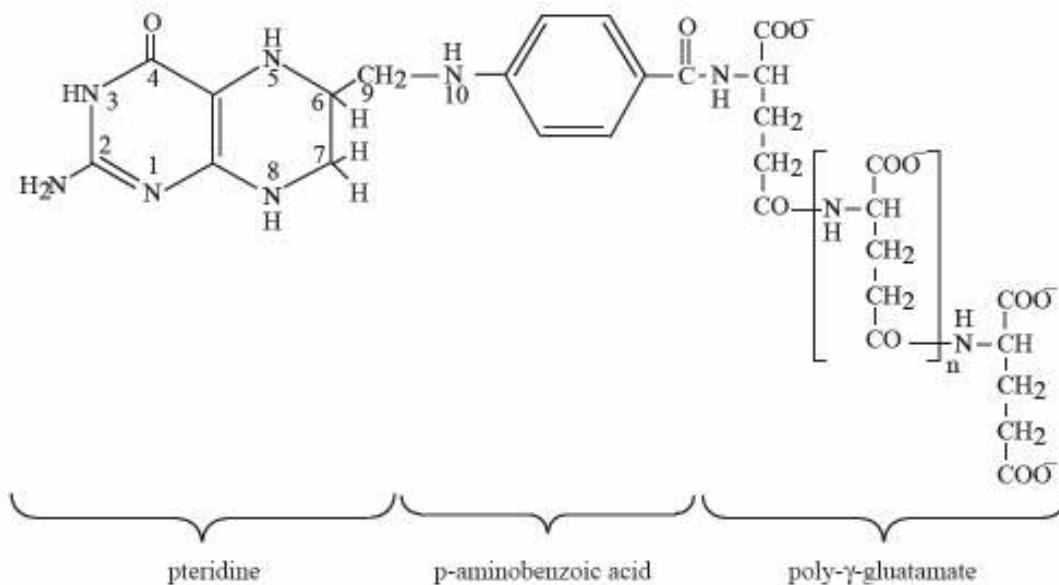


Figure 1.1 Structure of Tetrahydrofolate. THF consists of a pteridine ring attached to p-aminobenzoic acid and glutamate residues. Activated one-carbon units are carried at the N⁵ and/or N¹⁰ positions to produce 5-methyl, 5-formyl, 5,10-methylene (-CH₂-), 5,10-methenyl (=CH⁺-), and 10-formyl derivatives. The predominant forms of naturally occurring folates contain up to 8 glutamate residues.

1.2 Folate-mediated One-Carbon Metabolism

The major pathways of folate-mediated one-carbon metabolism are outlined in Figure 1.2. Although many of these reactions are shared by most cells, some are species- or tissue- or organelle-specific. The enzyme dihydrofolate reductase (DHFR, reaction 10) reduces folate and 7,8-dihydrofolate (DHF) to 5,6,7,8-tetrahydrofolate (THF). The major source of one-carbon units for most organisms is carbon 3 of serine, which is synthesized from carbohydrate precursors in the cytoplasm (Schirch,

1984). THF accepts a one-carbon unit from serine in a reaction catalyzed by serine hydroxymethyltransferase (SHMT, reaction 4) producing 5,10-methylene-THF and glycine. 5,10-methylene-THF is utilized in *de novo* thymidylate biosynthesis by thymidylate synthase (reaction 7) or reduced to 5-methyl-THF by 5,10-methylene-THF-reductase (reaction 5) for use in methionine biogenesis (reaction 6). It can also be oxidized to 10-formyl-THF in two sequential reactions catalyzed by 5,10-methylene-THF dehydrogenase (reaction 3) and 5,10-methenyl-THF cyclohydrolase (reaction 2). This form of the coenzyme is essential for *de novo* purine biosynthesis (reaction 8). 10-formyl-THF formation occurs also when formate is activated in an ATP-dependent reaction by 10-formyl-THF synthetase (reaction 1) releasing THF. Formate is produced by the oxidation of formaldehyde by formaldehyde dehydrogenase (reaction 11). THF can also be regenerated by the oxidation of 10-formyl-THF by 10-formyl-THF dehydrogenase (reaction 9). In prokaryotes, the interconverting activities of 5,10-methylene-THF to formate (5,10-methylene-THF dehydrogenase, 5,10-methenyl-THF cyclohydrolase, and 10-formyl-THF synthetase) are generally catalyzed by monofunctional proteins (Whitehead and Rabinowitz, 1986; Whitehead *et al.*, 1988). However, some bacteria such as *E. coli* (Dev *et al.*, 1978) and *Clostridium thermoaceticum* (O'Brien *et al.*, 1973) have a bifunctional cyclohydrolase/dehydrogenase. In eukaryotes, these three activities generally exist as part of a trifunctional protein called C₁-THF synthase (Paukert *et al.*, 1976; Tan *et al.*, 1977). The reversible interconversion of 5,10-methylene-THF to formate by C₁-THF

synthase is vital for the supply of activated one-carbon units required for the above mentioned cellular processes, thereby placing it in the center of one-carbon metabolism. In addition to serine, histidine (carbon 2 of the imidazole ring) catabolism and 5-formyl-THF can also supply activated one-carbon units at the level of 5,10-methenyl-THF (reactions 12 and 13, respectively) (Shane and Stokstad, 1984).

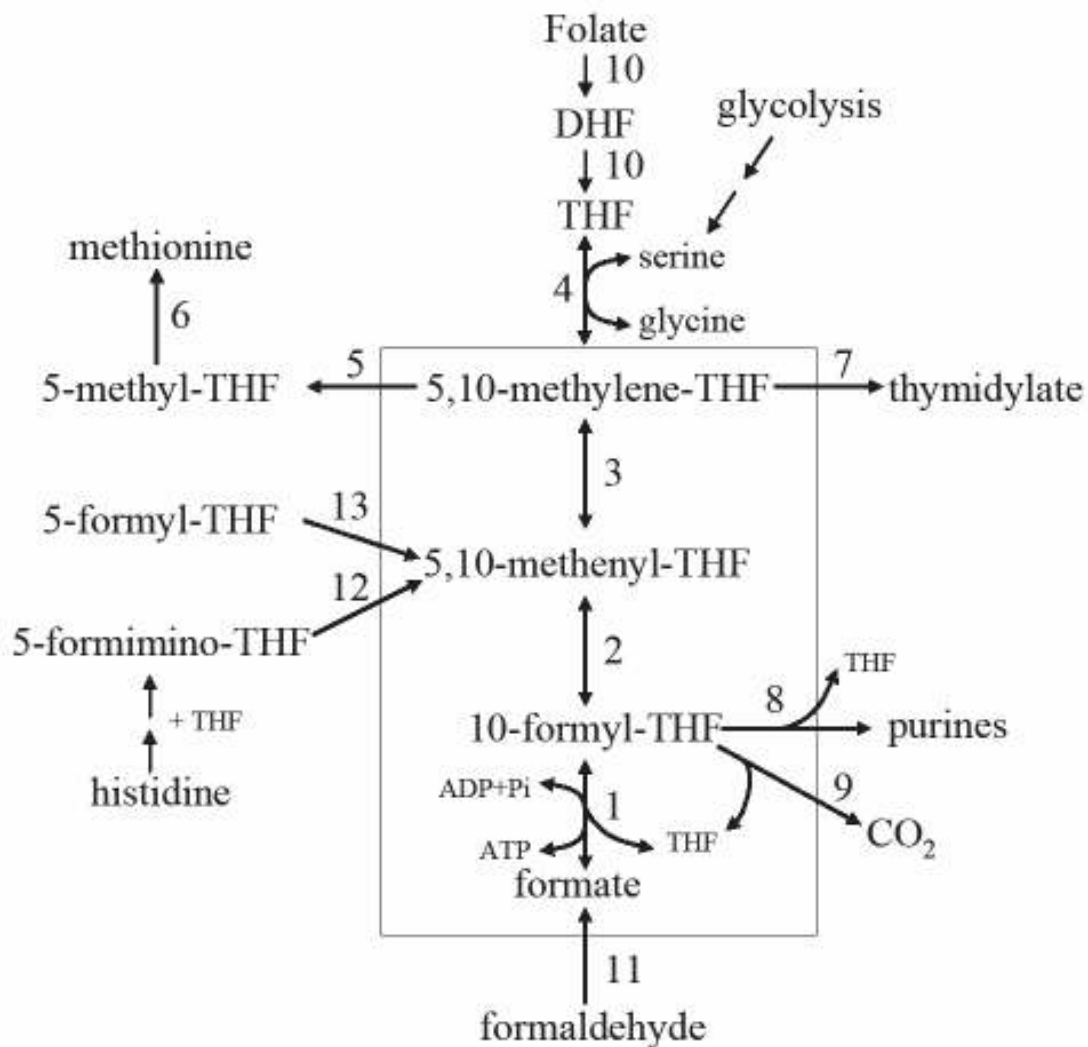


Figure 1.2 Major Pathways of Folate-dependent One-carbon Metabolism. Folate-mediated reactions that generate one-carbon units or utilize them for various cellular processes are shown. The reactions are catalyzed by **1**, 10-formyl-THF synthetase; **2**, 5,10-methenyl-THF cyclohydrolase; **3**, 5,10-methylene-THF dehydrogenase; **4**, serine hydroxymethyltransferase; **5**, 5,10-methylene-THF reductase; **6**, methionine synthase; **7**, thymidylate synthase; **8**, GAR transformylase and AICAR transformylase; **9**, 10-formyl-THF dehydrogenase; **10**, dihydrofolate reductase; **11**, formaldehyde dehydrogenase; **12**, 5-formimino-THF deaminase; **13**, methenyl-THF synthetase. Enzyme activities **1**, **2**, and **3** (inside the box) are part of trifunctional protein, C₁-THF synthase, which is the focus of this dissertation.

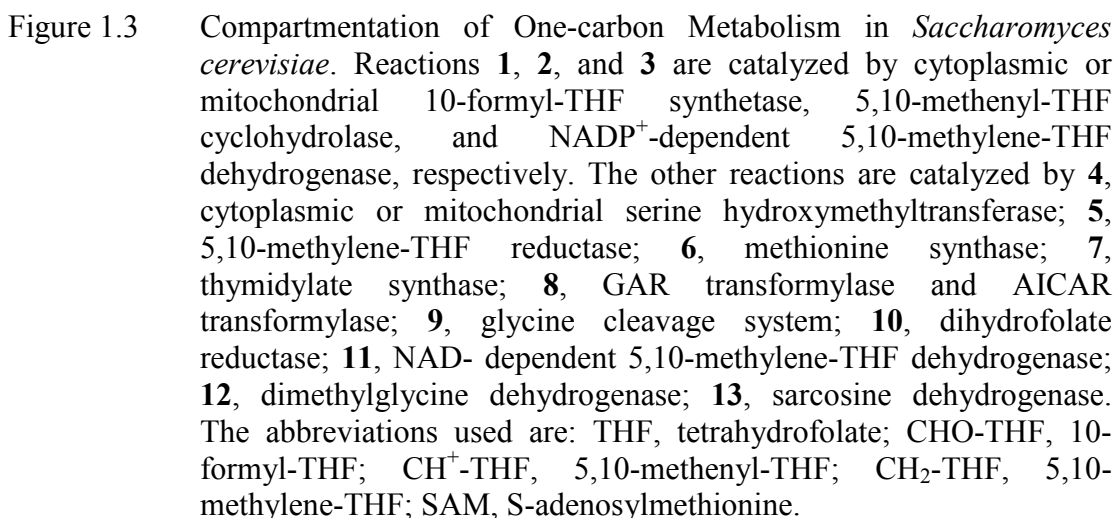
1.3 Compartmentation of Folate-mediated One-Carbon Metabolism

Folate-mediated one-carbon metabolism is an example of compartmentation within the intracellular organelles so that the intermediates of the pathway can serve distinct purposes (Appling, 1991). In eukaryotes, a parallel set of one-carbon unit interconverting activities are present both in the cytoplasm and in the mitochondria (Figure 1.3). In the yeast, *Saccharomyces cerevisiae*, serine hydroxymethyltransferase (SHMT) isozymes are present in both the cytoplasm (*SHM2* gene) and mitochondria (*SHM1* gene) (McNeil et al, 1994). Also, the C₁-THF synthase enzyme is encoded by the *ADE3* gene in the cytoplasm and the *MIS1* gene in the mitochondria (Paukert *et al.*, 1977; Shannon and Rabinowitz, 1986; Staben and Rabinowitz, 1986). The dehydrogenase activity that exists as part of the trifunctional enzyme is NADP⁺ dependent (Staben and Rabinowitz, 1986; Shannon and Rabinowitz, 1988). A monofunctional cytoplasmic NAD-dependent 5,10-methylene-THF dehydrogenase has also been isolated and characterized in *Saccharomyces cerevisiae* (Barlowe and Appling, 1990), encoded by the *MTD1* gene (West *et al.*, 1993). In mammals, the existence of a cytoplasmic C₁-THF synthase has been known for several years (Paukert *et al.*, 1976). The cytoplasmic as well as mitochondrial isozymes of SHMT have been characterized in rabbit liver (Martini *et al.*, 1987 and Martini et al., 1989) and humans (Garrow *et al.*, 1993; Stover *et al.*, 1997). A bifunctional mitochondrial cyclohydrolase/dehydrogenase has also been isolated from transformed cells (Mejia and MacKenzie, 1985; Mejia and MacKenzie,

1988). Dihydrofolate reductase and folylpolyglutamate synthetase activities are present in both the cytoplasm and mitochondria of yeast (Zelikson and Luzzati, 1977) and mammalian cells (Shane, 1989).

In addition to the above mentioned activities, there are some folate-dependent enzymes that are exclusively mitochondrial: glycine cleavage system (GCS) (reaction 9) (Motokawa and Kikuchi, 1971), dimethylglycine dehydrogenase (reaction 12), and sarcosine dehydrogenase (reaction 13) (Frisell *et al.*, 1965). The glycine cleavage system permits the donation of a one-carbon unit from glycine to THF producing 5,10-methylene-THF (Kikuchi, 1973), which is then oxidized to 10-formyl-THF. Mitochondrial protein synthesis uses this form of the folate coenzyme for formylation of initiator transfer RNA (Galper and Darnell, 1969; Bianchetti *et al.*, 1977; Staben and Rabinowitz, 1984). 5,10-methylene THF can also be utilized in thymidylate biosynthesis or methionine biogenesis. The mitochondrial pool of folates is taken up from the cytoplasm. Although THF and its derivatives are not transported to the mitochondria to any significant extent, folate and dihydrofolate are readily imported (Cybulski and Fisher, 1981). In fact, studies on glycine auxotrophs of Chinese Hamster Ovary (CHO) cells have led to the discovery of a mitochondrial inner membrane folate transporter (Titus and Moran, 2000). In mitochondria, serine and glycine, which are readily transported across the inner membrane (Cybulski and Fisher, 1976), provide a supply of one-carbon units. Also, dimethylglycine and sarcosine can supply one-carbon units at the level of 5,10-methylene-THF catalyzed

by dimethylglycine dehydrogenase and sarcosine dehydrogenase, respectively, in mammals (Wittwer and Wagner, 1981). The source for dimethylglycine is betaine, which is produced by choline oxidation, and dimethylglycine is further metabolized to sarcosine (Lewis *et al.*, 1978).



1.4 Role of Mitochondrial One-carbon Metabolism in Cytoplasmic Processes

There is accumulating evidence suggesting the significance of mitochondrial folate-mediated one-carbon metabolism in supporting cytoplasmic processes. A yeast mutant auxotrophic for thymidylate, methionine, adenine, and histidine was found to be deficient in three mitochondrial enzyme activities, serine hydroxymethyltransferase, thymidylate synthase, and dihydrofolate reductase, while having the corresponding cytoplasmic activities (Zelikson and Luzzati, 1977). Also, yeast mutants lacking cytoplasmic 10-formyl-THF synthetase are purine auxotrophs despite retaining all the cytoplasmic activities necessary to synthesize 10-formyl-THF from serine (McKenzie and Jones, 1977). At the same time, cytoplasmic 5,10-methylene-THF dehydrogenase and 5,10-methenyl-THF cyclohydrolase activities are dispensable in the synthesis of purines using serine as a one-carbon source in some other yeast mutants (McKenzie and Jones, 1977). In CHO cells, several glycine-requiring mutants were discovered by chemical mutagenesis (Kao *et al.*, 1969; McBurney and Whitmore, 1974; Kao and Puck, 1975). They were classified into four complementation groups, GLYA, GLYB, GLYC, and AUXB1. Among these, GLYA exhibits mitochondrial serine hydroxymethyltransferase deficiency while possessing its active cytoplasmic counterpart (Chasin *et al.*, 1974). Experiments with GLYA cells showed that mitochondrial serine hydroxymethyltransferase was responsible for regulating glycine concentration under normal cellular conditions (Pfendner and Pizer, 1980). The cytoplasmic isozyme by itself did not support glycine requirements

of the cell. GLYB, GLYC, and AUXB1 are all auxotrophic for glycine, though the level of cytoplasmic and mitochondrial serine hydroxymethyltransferase is normal unlike GLYA (Taylor and Hanna, 1982). GLYB and GLYC are also deficient in mitochondrial folylpolyglutamates, while having normal amounts of mitochondrial folylpolyglutamate synthetase. In GLYB, the glycine requirement was shown to be due to deficiency of a mitochondrial inner membrane transport protein for folates (Titus and Moran, 2000). In contrast to the other glycine auxotrophs, AUXB1 was found to be auxotrophic for adenosine and thymidine as well (McBurney and Whitmore, 1974). A mutation in the structural gene for folylpolyglutamate synthetase is responsible for these multiple requirements in AUXB1 (Taylor and Hanna, 1977; Taylor and Hanna, 1979). This mutation resulted in monoglutamate forms of the folate pool (Foo and Shane, 1982). The glycine auxotrophy was eliminated in AUXB1 transfectants expressing human folylpolyglutamate synthetase (Lowe *et al.*, 1993). However, AUXB1 transfectants expressing *E. coli* folylpolyglutamate synthetase (FPGS) were not able to eliminate the glycine auxotrophy since the FPGS activity was not localized in mitochondria (Lin *et al.*, 1993). When the *E. coli* folylpolyglutamate synthetase was targeted to the mitochondria, the mitochondrial folate pool was converted to polyglutamate forms, thereby removing the glycine dependence (Lin and Shane, 1994). The above experiments suggest that mitochondrial SHMT is essential for glycine synthesis in CHO cells and is dependent on the polyglutamate forms of THF. It also provides

evidence for the involvement of mitochondrial folate-dependent reactions in cytoplasmic processes.

Based on the above observations, a new pathway was proposed by our laboratory for one-carbon metabolism wherein mitochondria were significantly involved in the generation of active one-carbon units to be used in cytoplasmic processes (Figure 1.4). The hypothesis suggests that serine enters the mitochondria from the cytoplasm and donates its one-carbon units to THF forming 5,10-methylene-THF, catalyzed by mitochondrial SHMT (reaction 4). 5,10-methylene-THF is converted to formate in three reactions catalyzed by mitochondrial C₁-THF synthase (mitochondrial reactions 3, 2, and 1). The intermediate product, 10-formyl-THF, can be utilized in formylation of initiator tRNA to be used in mitochondrial protein synthesis (reaction 7). Formate produced in the mitochondria exits into the cytoplasm where 10-formyl-THF synthetase, in an ATP-dependent reaction (cytoplasmic reaction 1), converts it into 10-formyl-THF for purine biosynthesis.

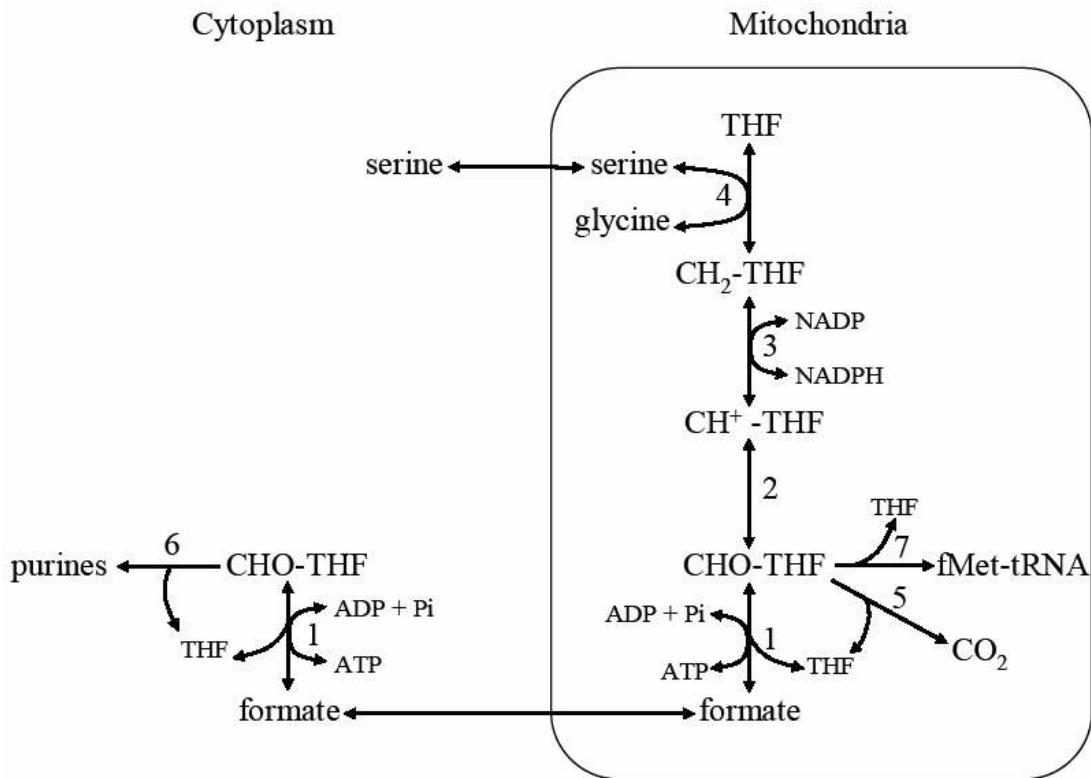


Figure 1.4 Proposed Mammalian One-carbon Cycle. Reactions **1**, **2**, and **3** are catalyzed by mitochondrial 10-formyl-THF synthetase, 5,10-methenyl-THF cyclohydrolase, and 5,10-methylene-THF dehydrogenase, respectively. The other reactions are catalyzed by **4**, serine hydroxymethyltransferase; **5**, 10-formyl-THF dehydrogenase; **6**, GAR transformylase and AICAR transformylase; **7**, methionyl-tRNA formyltransferase. Abbreviations are as in Figure 1.3.

This hypothesis was supported by experiments performed on rat liver mitochondria in our lab. In 1988, the ability of intact mitochondria to metabolize L-[3-¹⁴C]serine and [¹⁴CH₃]sarcosine to [¹⁴C]formate was demonstrated (Barlowe and

Appling, 1988). The formate produced was rapidly released from the mitochondria. In eukaryotic mitochondria, there is an enzyme, 10-formyl-THF dehydrogenase, which can oxidize 10-formyl-THF into CO₂ and THF, via an NADP-dependent dehydrogenase activity (Figure 1.4; *reaction 5*). This enzyme also has a 10-formyl-THF hydrolase activity, at least *in vitro*, which can hydrolyze 10-formyl-THF into formate and THF (Kutzbach and Stokstad, 1968). The 10-formyl-THF dehydrogenase activity was detected in the cytoplasmic and mitochondrial fractions. However, only a low level of hydrolase activity was detected in the mitochondria, arguing against the possibility that this activity was contributing to formate production in the mitochondria.

In another study using actively respiring intact rat liver mitochondria, formate production from L-[3-¹⁴C]serine was shown to be dependent on high ADP level and the oxidized NADP⁺/NADPH pool, supporting the involvement of a mitochondrial C₁-THF synthase (Garcia-Martinez and Appling, 1993). No cofactors were added externally showing that the conversion of L-[3-¹⁴C]serine to [¹⁴C]formate solely used endogenous mitochondrial cofactor pools. When the experiment was done using sonicated mitochondria, formate production became dependent on the external addition of NADP⁺, ADP, and polyglutamylated THF, providing further evidence for the proposed pathway. Many years earlier, a Japanese group had identified an unknown one-carbon compound being produced and released from avian mitochondria, which they proposed to be formate, and being utilized in cytoplasmic

purine biosynthesis (Yoshida and Kikuchi, 1971). Studies conducted in our lab showed that, similar to the avian system, rat liver mitochondria could produce formate to be used in cytoplasmic purine biosynthesis (Barlowe and Appling, 1988; Garcia-Martinez and Appling, 1993). These studies support the proposal that THF-dependent serine oxidation into formate is the significant pathway for mitochondrial flux of one-carbon units.

1.5 Importance of Mammalian Mitochondrial C₁-THF synthase

Since C₁-THF synthase is essential for the interconversion of 5,10-methylene-THF to formate, many metabolic processes in the mitochondria may depend on this enzyme. As mentioned above, mitochondrial protein synthesis uses formylation of initiator Met-tRNA by 10-formyl-THF and methyl groups from oxidation of choline enter the one-carbon cycle at the level of 5,10-methylene-THF. The metabolism of glycine via glycine cleavage system (GCS) (Figure 1.3; *reaction 9*) is strictly a mitochondrial process. Studies done on patients with nonketotic hyperglycinemia, a genetic disease with defects in the GCS system causing high glycine concentrations (Hamosh *et al.*, 1995), suggested that GCS and homocysteine metabolism may be related. Since glycine cleavage supplies one-carbon units as 5,10-methylene-THF, defects in GCS also result in a shortage of mitochondrial one-carbon units. Elevated plasma homocysteine level was found in these patients and they responded to treatment with 5-formyl-THF (Van Hove *et al.*, 1998; Randak *et al.*, 2000), pointing

out that homocysteine remethylation was defective. It could be postulated that mitochondrial formate production was defective in these patients, in turn resulting in a deficiency of one-carbon units for cytoplasmic processes like methionine biogenesis. *In vitro* as well as *in vivo* experiments done in our lab on rat liver mitochondria (Barlowe and Appling, 1988; Garcia-Martinez and Appling, 1993) and yeast (Pasternack *et al.*, 1994; West *et al.*, 1996) have already provided evidence for mitochondrial formate production and its transport and utilization in the cytoplasmic processes. In addition to that, a stable isotope study done in humans using deuterated serine ($[^2\text{H}_3]$ serine) showed that together with the cytoplasm, mitochondria also supply one-carbon units for methionine biogenesis (Gregory *et al.*, 2000). This study supports the hypothesis that in mammals, formate produced in the mitochondria provides one-carbon units for cytoplasmic processes and suggests a major role for mitochondrial C_1 -THF synthase in one-carbon cycle.

1.6 The Domain Structure of C_1 -THF Synthase

In *Saccharomyces cerevisiae*, both the cytoplasmic and mitochondrial isozymes of C_1 -THF synthase exist as homodimers of 100 kDa subunits. Each subunit consists of a C-terminal 10-formyl-THF synthetase domain of ≈ 70 kDa and an N-terminal bifunctional dehydrogenase/cyclohydrolase domain of ≈ 30 kDa linked via a proteolytically sensitive connector region (Figure 1.5). This subunit size and domain structure is shared by cytoplasmic isozymes from mammalian and avian

sources (Paukert *et al.*, 1976; Tan and MacKenzie, 1977; Tan and MacKenzie, 1979; Smith *et al.*, 1980; Villar *et al.*, 1985; Cheek and Appling, 1989; Hum and MacKenzie, 1991).

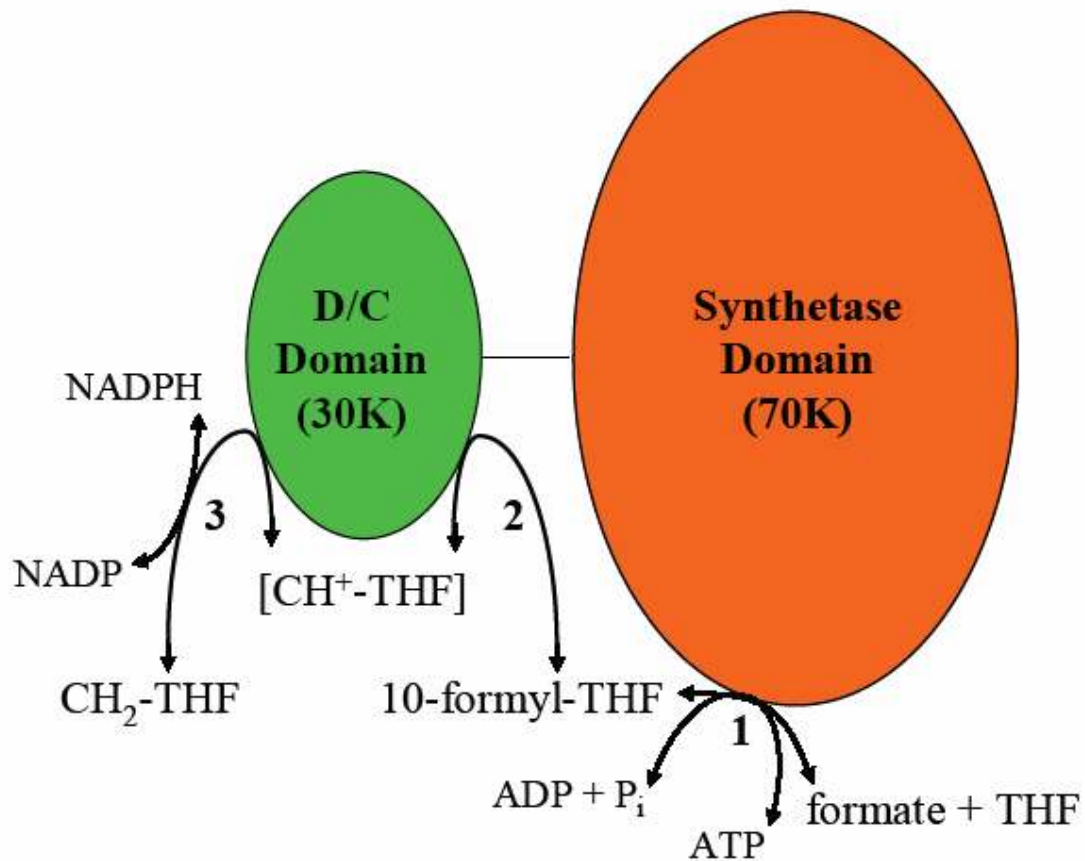


Figure 1.5 Domain Structure of C₁-THF Synthase. C₁-THF synthase is a homodimer of 100 kDa subunits. Each subunit has a C-terminal synthetase domain of ~70 kDa and an N-terminal dehydrogenase/cyclohydrolase (D/C) domain of ~30 kDa linked through a proteolytically sensitive connector region. Reactions 1, 2, and 3 are: 10-formyl-THF synthetase, 5,10-methenyl-THF cyclohydrolase, and 5,10-methylene-THF dehydrogenase, respectively.

1.7 Research History of Mammalian Cytoplasmic and Mitochondrial

C₁-THF Synthase

The cytoplasmic C₁-THF synthase in mammals has been isolated from various sources including rabbit liver (Villar *et al.*, 1985), sheep liver (Paukert *et al.*, 1976) and porcine liver (Tan *et al.*, 1977; Schirch, 1978). The human cytoplasmic C₁-THF synthase cDNA (2805-bp) was isolated by screening a λ gt10 human colonic adenocarcinoma cell line LS-180 cDNA library (Hum *et al.*, 1988). The purified protein (~ 101 kDa) is a single polypeptide that contains the activities of 5,10-methylene-THF dehydrogenase, 5,10-methenyl-THF cyclohydrolase and 10-formyl-THF synthetase. The cDNA encoding the rat cytoplasmic C₁-THF synthase has been isolated and characterized as well (Thigpen *et al.*, 1990). The dehydrogenase activity of the trifunctional enzyme is NADP-dependent in all mammals studied so far.

However, the existence of C₁-THF synthase in mammalian mitochondria has been controversial. In 1960, an NAD-dependent 5,10-methylene-THF dehydrogenase activity, which required Mg²⁺, was reported in Ehrlich ascites tumor cells (Scrimgeour and Huennekens, 1960). Later, MacKenzie and coworkers confirmed this activity in Ehrlich ascites tumor cells as well as in transformed mammalian cell lines, and embryonic rat liver (Mejia and MacKenzie, 1985). Among adult differentiated tissues, high activity was observed only in bone marrow, whereas spleen and thymus showed very low activity. Similar to the trifunctional enzyme, the dehydrogenase activity was found along with a cyclohydrolase activity, and the

purified bifunctional enzyme had a molecular weight of 34 kDa. In contrast to the cytoplasmic enzyme, this activity required Mg^{2+} and inorganic phosphate (Mejia *et al.*, 1986), both of which were later shown to be involved in NAD binding (Rios-Orlandi and MacKenzie, 1988; Yang and MacKenzie, 1993). There are other NAD-dependent dehydrogenases isolated from *Clostridium formicoaceticum* (Moore *et al.*, 1974), *Acetobacterium woodii* (Tanner *et al.*, 1978), and *Saccharomyces cerevisiae* (Barlowe and Appling, 1990), but they are all monofunctional. The bifunctional NAD-dependent 5,10-methylene-THF dehydrogenase/5,10-methenyl-THF cyclohydrolase was shown to be mitochondrial using immunolocalization experiments (Mejia and MacKenzie, 1988). Analysis of murine cDNA clones showed that the bifunctional enzyme is nuclear encoded, and has an N-terminal extension with characteristic features of a mitochondrial targeting sequence (Belanger and MacKenzie, 1989). Based on sequence alignment with the known trifunctional enzymes, the bifunctional enzyme exhibits high homology with the NADP-dependent yeast mitochondrial enzyme (45%), and also with the cytosolic enzymes in humans (40%) and in yeast (36%). Mejia and MacKenzie had reported NAD-dependent dehydrogenase activity in bone marrow (Mejia and MacKenzie, 1985), but studies by another group showed no such dehydrogenase activity (Smith *et al.*, 1990). In their hands, only rat adrenal cortex showed NAD-dependent dehydrogenase activity (Smith *et al.*, 1990), though the mRNA encoding this enzyme was reported to be present at low levels in all tissues examined (Peri and MacKenzie, 1993). A knockout of the

gene encoding the bifunctional enzyme in mice resulted in embryonic lethality (Di Pietro *et al.*, 2002) and embryonic fibroblasts obtained from the knockout mice were glycine auxotrophs (Patel *et al.*, 2003). These authors suggested that the metabolic role of the bifunctional enzyme was to support purine biosynthesis by providing one-carbon units in the form of 10-formyl-THF. Based on their studies, MacKenzie and coworkers have argued that mammalian mitochondria lack a C₁-THF synthase and that the bifunctional NAD-dependent dehydrogenase/cyclohydrolase is the mammalian homolog of the trifunctional yeast mitochondrial enzyme (Yang and MacKenzie, 1993; Di Pietro *et al.*, 2002).

1.8 Current Study

Based on the results from rat liver mitochondrial experiments (Barlowe and Appling, 1988; Garcia-Martinez and Appling, 1993), the Appling lab has hypothesized that mammalian mitochondria possess a trifunctional C₁-THF synthase capable of converting 5,10-methylene-THF into formate (Figure 1.4, *mitochondrial reactions 3, 2, and 1*). In the beginning of 2000, a BLAST search done by Dr. Appling using the cDNA sequence of the human cytoplasmic C₁-THF synthase (Hum *et al.*, 1988) resulted in the identification of a putative human mitochondrial C₁-THF synthase partial cDNA clone (DKFZp586G1517). This clone was constructed by the German Genome Project (RZPD German Research Center for Genome Research) (Wiemann *et al.*, 2001) and deposited in the GenBankTM/EBI Data Bank (accession

number AL117452). The truncated cDNA clone was purchased from RZPD, and was determined that it was missing a 5' exon.

This dissertation focuses on the full-length cDNA cloning and characterization of the human mitochondrial C₁-THF synthase. Chapter 2 describes the construction of a full-length cDNA clone by splice overlap extension (SOE)-PCR and its cloning, expression, and immunolocalization in Chinese hamster ovary cells. Chapter 3 describes the tissue distribution of the mRNA and transcript mapping of the 5' end. Chapter 4 describes transcript mapping of the 3' end of a short transcript (seen in Northern blots) and cDNA cloning, and purification of the short isoform for polyclonal antibody production. Chapter 5 describes the sublocalization of human mitochondrial C₁-THF synthase followed by a summary of the significance of our study in relation to a better understanding of folate-dependent one-carbon metabolism in mammalian mitochondria.

CHAPTER 2

cDNA Cloning and Immunolocalization of Human Mitochondrial C₁-THF

Synthase in Chinese Hamster Ovary (CHO) Cells

2.1 OVERVIEW

In this chapter, the identification and characterization of the human gene encoding a mitochondrial C₁-THF synthase is described. The construction of full-length cDNA (2934 base pairs (bp)), its cloning into the mammalian expression vector, pcDNA3.1D/V5-His-TOPO, and immunolocalization of the recombinant protein in CHO cells is described. The gene spans 236 kilobase pairs (kbp) on human chromosome 6 and encodes a protein of 978 amino acids, including an N-terminal mitochondrial targeting sequence. The mitochondrial isozyme is 61% identical to the cytoplasmic isozyme. Splice overlap extension (SOE)-PCR was used to generate the full-length cDNA, which was cloned into the mammalian expression vector pcDNA3.1D/V5-His-TOPO using TOPO cloning. The full-length cDNA fused to a C-terminal V5 epitope tag was expressed in CHO cells. Subcellular localization of the recombinant protein was determined by immunoblotting using antibodies against the V5 epitope. A signal at ~ 107 kDa was detected only in the mitochondrial fraction of transfected CHO cells thereby confirming that this cDNA encodes a protein that localizes exclusively to the mitochondria in a mammalian cell line.

Portions of this work have been published in the Journal of Biological Chemistry in the issue of October 31st, 2003 (Prasannan, P., Pike, S., Peng, K., Shane, B. and Appling, D.R. (2003) *J. Biol. Chem.* **278**, 43178-43187) and these are identifiable by quotation marks and increased indent.

2.2 EXPERIMENTAL PROCEDURES

2.2.1 cDNA Identification

The experiments described in sections 2.2.1 and 2.2.2 were carried out by Dr. Appling several years ago.

“A partial cDNA clone (DKFZp586G1517) constructed by the German Genome Project (RZPD German Research Center for Genome Research; Wiemann *et al.*, 2001) was identified in the GenBank™/EBI Data Bank (accession number AL117452 [[GenBank](#)]) by a BLAST search using the cDNA sequence of the human cytoplasmic C₁-THF synthase (Hum *et al.*, 1988). This cDNA contained 390 nucleotides (nt) of 3'-noncoding sequence and a poly(A) tail, but lacked a start codon, indicating that it was truncated at the 5'-end. The truncated cDNA clone (in pSPORT 1 *E. coli* cloning vector) was obtained from RZPD, and its sequence was confirmed by sequencing at the DNA Analysis Facility of the University of Texas (Austin, TX). The Human Genome Database contained the entire gene corresponding to this

cDNA and predicted an additional 5'-exon that encoded 60 additional N-terminal amino acids”.

Four primers were designed to construct the full-length human mitochondrial C₁-THF synthase cDNA that could be cloned into the mammalian expression vector, pcDNA3.1D/V5-His-TOPO.

a) **TOPO 5' (5'-CACCATGGGCACGCGTCTGCCGCTC-3')**

It was used as the forward primer in PCR amplification of exon 1 as well as in SOE-PCR. The 5' CACC sequence enabled TOPO cloning into the vector. The ATG start codon is underlined.

b) **TOPO 3' (5'-GAACAAGCCTTTAACTTGTTCTGTTTC-3')**

It was used as the reverse primer in PCR amplification of partial cDNA as well as in SOE-PCR. It is complementary to the last nine codons of the open reading frame before the stop codon. The stop codon was not included in the primer to enable cloning of the ORF in frame with the V5 epitope and His₆ tag at the C terminus.

c) **HumitoSOE3' (5'-CTTCTCTGACGATGGAGTCCCG-3') & GS5'SOE (5'-GGGACTCCATCGTCAGAGAAG-3')**

HumitoSOE3' and GS5'SOE were used as the reverse and forward primers for PCR amplification of exon 1 and partial cDNA, respectively. They are complementary to each other (except for one nucleotide at the 3'-end of

humitoSOE3'), which enabled SOE-PCR of exon 1 and partial cDNA to produce the full-length cDNA.

2.2.2 PCR Amplification of 5'-exon (Exon 1)

The missing 5'-exon (exon 1) was PCR-amplified from a genomic P1 artificial chromosome clone (dJ44A20) obtained from the Sanger Centre (Cambridge, UK) using primers DRA1(5'-GTGTCCCCTGAGAACCAGC-3') and INTRON1 (5'-CCGACACTCACCTGACGATGGA-3'). The PCR-amplified product was gel-purified using a QIAquick gel extraction kit (QIAGEN), subcloned into the pGEM-T Easy vector (Promega), and its sequence was verified. The resulting exon 1 clone was used as template to amplify the 230-bp exon 1 fragment by PCR. The primers used were TOPO 5' and humitoSOE 3'. The PCR product was run on a 0.8 % agarose gel and gel-purified using a QIAquick gel extraction kit. Due to the high GC content of exon 1, it was necessary to use MasterAmp *Tfl* DNA polymerase (Epicentre) in the PCRs.

2.2.3 PCR Amplification of Partial cDNA

A partial cDNA clone (DKFZp586G1517) constructed by the German Genome Project was used as a template to amplify the truncated cDNA. The 2719-bp cDNA fragment was amplified with *Pfu* polymerase (Stratagene) and primers

GS5'SOE and TOPO 3'. The PCR product was run on a 0.8 % agarose gel and gel-purified using a QIAquick gel extraction kit.

2.2.4 SOE-PCR to generate Full-length Human Mitochondrial C₁-THF Synthase cDNA

SOE-PCR (Horton *et al.*, 1993) was applied to create full-length human mitochondrial C₁-THF synthase cDNA. The 230-bp and 2719-bp PCR products served as templates in an SOE-PCR with *Tfl* polymerase, and primers TOPO5' and TOPO3' to get the full-length cDNA product (2934-bp) (Figure 2.1) and it was gel-purified using a QIAquick gel extraction kit.

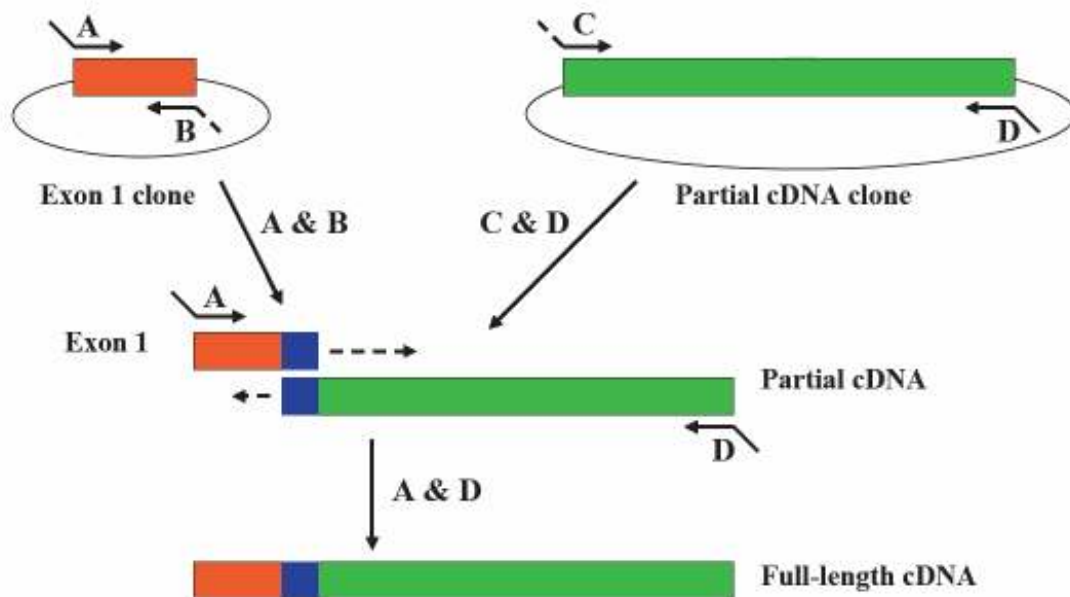


Figure 2.1 SOE-PCR to create Full-length Human Mitochondrial C₁-THF synthase cDNA. Exon 1 and partial cDNA were PCR-amplified separately from an exon 1 clone and a partial cDNA clone, respectively. Primers B and C were designed to give PCR products with overlapping ends (shown as gray boxes) so that they act as primers for each other to give the spliced product, which gets further amplified by primers A and D to yield the full-length cDNA. Primers are: A, TOPO 5'; B, humitoSOE3'; C, GS5'SOE; D, TOPO 3'.

2.2.5 TOPO Cloning of Full-length cDNA

The full-length cDNA product (2934-bp) was cloned into the mammalian expression vector pcDNA3.1D/V5-His-TOPO (5514-bp; Invitrogen) using directional TOPO cloning. TOPO cloning is a technique used for directional cloning of blunt

end PCR product into a mammalian expression vector (Figure 2.2). In the pcDNA3.1D/V5-His-TOPO expression system, topoisomerase I from *Vaccinia* virus is used to cleave one strand of the vector after 5'-CCCTT sequence favoring the formation of a covalent bond between the 3' phosphate of the cleaved strand and a tyrosyl residue of topoisomerase I. In addition to that, to enable directional cloning, the vector also has a single stranded overhang (GTGG) added to its 5'-end. Also, the forward primer used to amplify the insert (TOPO 5') was designed to have a CACCATG (sequence complementary to the overhang is underlined) sequence at its 5'-end that facilitates directional cloning and forms a Kozak consensus sequence (CACCATGG) (Kozak, 1987). When mixed together, the GTGG overhang in the vector attacks the 5'-end of the insert and displaces the bottom strand of the insert. The 5' hydroxyl group of the insert then attacks the phospho-tyrosyl bond between the vector and topoisomerase forming a phosphodiester bond and releases the enzyme. In addition to TOPO cloning site, the vector has a human cytomegalovirus (CMV) promoter for high level expression of the recombinant protein in mammalian cells, a C-terminal V5 epitope for detection of recombinant protein using anti-V5 antibodies, and ampicillin and neomycin resistance gene for selection of vector in *E. coli* and of stable transfectants in mammalian cells, respectively.

TOPO cloning reaction and transformation were performed according to pcDNA3.1 Directional TOPO Expression kit instructions (Invitrogen) (Appendix IV). The transformants were screened by PCR with a vector primer (T₇) and a gene

specific primer, and positive plasmids (pcDNA3.1-humito) were prepared using a QIAprep Spin Miniprep kit (QIAGEN). Sequencing was done on the plasmid using vector-specific and gene-specific primers at the DNA facility of The Institute of Cellular and Molecular Biology at the University of Texas at Austin. The results showed that there was a base substitution in the full-length clone at nucleotide 211 (C → T) compared with the original cDNA and genomic sequences, presumably incorporated during PCRs. *Tfl* polymerase, which was used in PCRs due to the high GC content of exon 1 lacks 3' → 5' proof reading activity. This substitution that would have changed the codon CGG to AGG in the cDNA (amino acid Arg to Trp in the encoded protein) was repaired using the QuikChange site-directed mutagenesis kit (Stratagene).

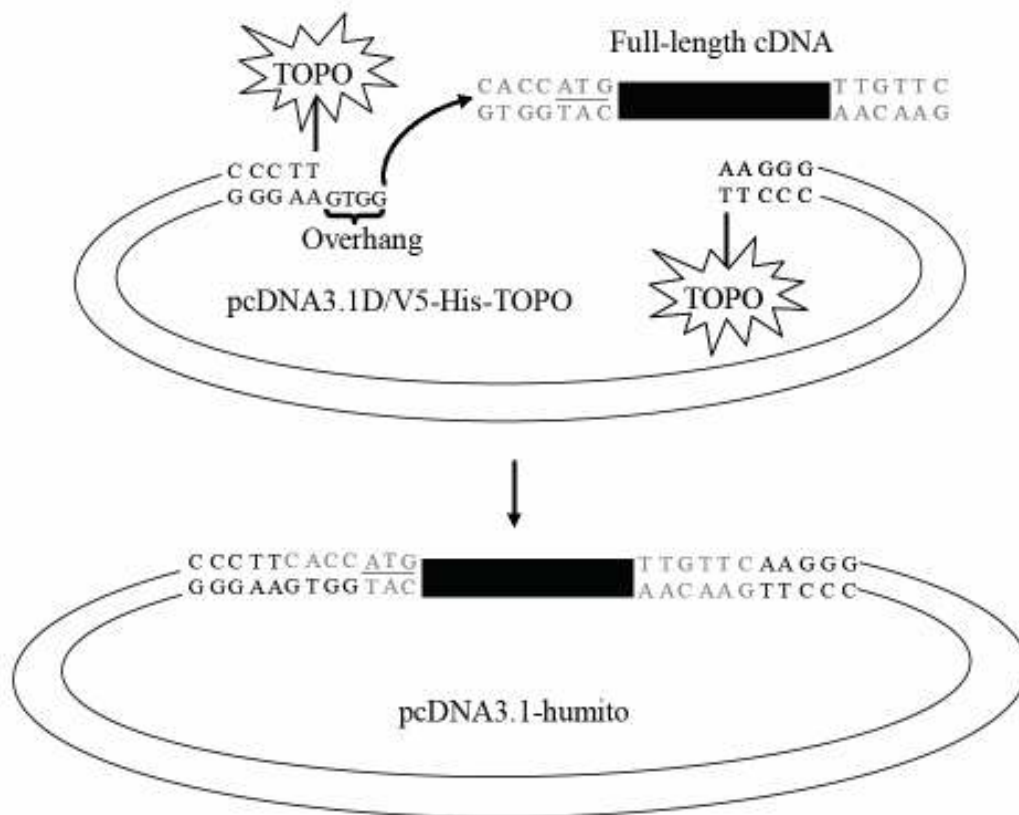


Figure 2.2 TOPO Cloning of Full-length cDNA into pcDNA3.1D/V5-His-TOPO Vector. See section 2.2.5 for details. The ATG start codon in the full-length cDNA is shown underlined. TOPO, Topoisomerase I.

2.2.6 Site Directed Mutagenesis

The QuikChange site-directed mutagenesis kit was used to mutate the ‘T’ at position 211 to ‘C’. This method makes use of two oligonucleotide primers that are complementary to each other containing the desired mutation and *Pfu* DNA polymerase. The primers bind to the dsDNA template that is undergoing repair, and

are extended by PCR creating the mutated plasmid with staggered nicks. The resulting PCR product is restriction digested with *Dpn* I endonuclease (recognition sequence-5'-Gm⁶ATC-3') that will digest only the methylated parental DNA template (DNA isolated from *dam*⁺ *E. coli* strain is methylated). The mutated plasmid is transformed into RbCl-competent *E. coli* XL1-Blue (Stratagene) (Appendix IV) cells and a plasmid miniprep is done on the colonies obtained.

Two primers were designed as per kit instructions to create the mutated plasmid by PCR.

PPMF (5'-CGCCCGCGGCGCGGGACTCCATC-3') & PPMR (5'-GATGGAGTCCCGCGCCGCGGGCG-3')

They both were complementary to each other that enabled binding to the opposite strands of pcDNA3.1-humito and covered nt +200 to +222 of the cDNA. The desired mutation to be achieved is shown underlined and is in the middle of the primer flanked by 11 correct bases.

2.2.7 Subcloning of pcDNA3.1-humito into pBluescript II KS (+/-)

A portion of the cDNA containing the base substitution was subcloned into a pBluescript II KS (+/-) (2961 bp; Stratagene) plasmid for site-directed mutagenesis. pcDNA3.1-humito was *Hind*III-digested and both the 816-bp and 7632-bp products were gel-purified using a QIAquick gel extraction kit. The larger fragment was saved at -20°C for later use. pBluescript II KS (+/-) was also *Hind*III-digested and

dephosphorylated using shrimp alkaline phosphatase. The plasmid DNA was then ligated with the 816-bp fragment using the Fast-Link™ DNA Ligation Kit (Epicentre) and the ligation reaction was transformed into RbCl-competent *E. coli* XL1-Blue cells. The positive colonies were selected on YT/agar (0.8% tryptone, 0.5% yeast extract, 0.5% NaCl, and 2% agar) plates containing 50µg/ml ampicillin and screened by PCR using M13 forward and M13 reverse vector primers. The positive plasmids (pBluescript II KS (+-)/pcDNA3.1-humito (816-bp); 3777-bp) were prepared using a QIAprep Spin Miniprep kit and screened again by HindIII digestion.

2.2.8 PCR Amplification of pBluescript II KS (+-)/pcDNA3.1-humito

About 50 ng template DNA was used to PCR-amplify the subclone using PPMF and PPMR as forward and reverse primers and *Pfu* DNA polymerase supplemented with betaine to enable amplification of the GC rich exon 1.

The PCR-amplified plasmid (3777-bp) was mixed with *Dpn* I (10U/50ng template DNA) and incubated at 37°C for 1 h to digest the parental supercoiled dsDNA. It was transformed into RbCl-competent *E. coli* XL1-Blue cells and positive colonies were selected on YT agar plates containing 50 µg/ml ampicillin. The positive plasmids were prepared using a QIAprep Spin Miniprep kit, sequenced using T₇ and T₃ vector primers, and the desired mutation was confirmed.

2.2.9 Subcloning of Mutated cDNA into pcDNA3.1-humito Larger Fragment

The *Hind*III-digested/gel-purified pcDNA3.1-humito larger fragment (see 2.2.7) was dephosphorylated using shrimp alkaline phosphatase. The mutated clone was *Hind*III-digested and the 816-bp fragment was gel-purified using a QIAquick gel extraction kit. They both were ligated using the Fast-LinkTM DNA Ligation Kit, transformed into RbCl-competent XL1-Blue cells, and positive colonies were selected on YT plates containing 50 µg/ml ampicillin. The colonies were screened by colony PCR with a vector primer and a gene-specific primer, and positive plasmids were prepared using a QIAGEN Plasmid Midi kit. The repaired full-length cDNA clone, pcDNA3.1-humito (Figure 2.3), was sequenced completely, and the correct sequence was confirmed (GenBankTM/EBI accession number AY374130).



Figure 2.3 Schematic Representation of pcDNA3.1-humito. The full-length human mitochondrial C₁-THF synthase cDNA was cloned into the mammalian expression vector, pcDNA3.1D/V5-His-TOPO. This construct has a V5 epitope and a His₆ tag at the C-terminus of the coding region. A cytomegalovirus (CMV) promoter drives the expression of the recombinant protein.

2.2.10 CHO Cell Transfection

CHO cell transfection and preparation of subcellular fractions (2.2.11) were performed by Dr. Kun Peng in Dr. Barry Shane's lab (University of California, Berkeley, CA). The procedures were done in the following way: CHO cells (1.5×10^5) were plated on 35-mm diameter dishes and cultured in α -minimal Eagle's medium (MEM) supplemented with 10% (v/v) fetal bovine serum (FBS). The cells were then transfected with 2 μ g of pcDNA3.1-humito/plate using the LipofectAMINE 2000 reagent method (Invitrogen). Briefly, 2 μ g of pcDNA3.1-humito was diluted into 100 μ l serum-free medium, OPTI-MEM, to give solution A. For solution B, 10 μ l Lipofectamine and 100 μ l OPTI-MEM were mixed. The two solutions, A & B, were mixed and incubated at room temperature for 45 min. The medium was removed from the 35-mm dishes containing cells to be transfected and the cells were

washed once with 2 ml serum-free medium. 0.8 ml of medium containing 5% FBS and 0.2 ml of solution A/B mix were applied to the cells and incubated at 37°C for ~6 h. After transfection, cells were cultured for an additional 48 h in regular medium before a geneticin (G418 Sulfate)-containing selective medium (0.8 mg/ml) was applied. The selective medium was applied for ~1 week until antibiotic-resistant colonies developed. Resistant colonies were picked, replated, cultured, and collected.

2.2.11 Preparation of Cell Homogenates and Subcellular Fractions

“Transfected cells were cultured in two 150-cm² T-flasks to yield 1–2 x 10⁸ cells. The monolayer was rinsed with phosphate-buffered saline (4 x 5 ml) at 4 °C and then incubated with phosphate-buffered saline containing 10 mM EDTA (10 ml) at room temperature until the cells detached (5–10 min). The flasks were tapped gently to dislodge the cells, and the cells were transferred to a 50-ml plastic conical tube. Cells were pelleted by centrifugation at 300 x g for 5 min at room temperature, and the cell pellet was washed with 15 ml of homogenization solution (HMS; 250 mM sucrose and 1 mM EDTA (pH 6.9)) at 4 °C. The cell pellet was resuspended in HMS (2 ml) at 4 °C, transferred to a Kontes nitrogen cavitation device, and exposed to a pressure of 36 p.s.i. for 30 min at 4°C. The suspension of disrupted cells was

collected into a 3-ml conical ground-glass Dual tissue grinder and further disrupted with four strokes of the homogenizer (Lin *et al*, 1993)".

"Nuclei and unbroken cells were sedimented by centrifugation at 900 x g for 6 min. The supernatant was removed carefully, transferred to another centrifuge tube, and stored on ice. The pellet was resuspended in HMS (1 ml) and further dispersed by four strokes in the grinder. After centrifugation at 900 x g for 6 min, the supernatant was combined with the first supernatant and stored on ice. The pellet was washed with HMS (3 x 1 ml), and the final viscous pellet (nuclear fraction) was resuspended in HMS (1 ml). The combined supernatants were centrifuged at 900 x g for 5 min, and the pellet was discarded. The volume of the supernatant (total post-nuclear supernatant fraction) was increased to 5 ml by the addition of HMS".

"The post-nuclear supernatant was centrifuged at 10,000 x g for 15 min, and the pellet was stored on ice. The supernatant was recentrifuged at 10,000 x g for 15 min to give a final supernatant (cytosolic fraction). The second pellet was combined with the first, washed with HMS (2 ml), and resuspended in HMS (1 ml) to give the mitochondrial fraction. Glutamate dehydrogenase activity (Schmidt, 1974) was used as a mitochondrial marker, and lactate dehydrogenase activity (Kornberg, 1955) was used as a cytoplasmic marker".

2.2.12 Immunoblotting

“The protein concentration of the cytosolic and mitochondrial fractions was determined using the Bradford assay (Bradford, 1976) with bovine serum albumin as a standard as given in appendix IV. Eighty µg of cytosolic and mitochondrial protein from transfected and untransfected CHO cells were electrophoresed by SDS-PAGE (Laemmli, 1970) on a 7.5% polyacrylamide gel for 50 min at 180 V. One-half of the gel was stained with Coomassie Brilliant Blue R 250 (USB), and the proteins on the other half were transferred onto a nitrocellulose membrane (Midwest Scientific) by electroblotting (Appendix IV). The primary antibody used for immunoprobining was mouse anti-V5 antibody (1:1000 dilution; Invitrogen) and the secondary antibody used was horseradish peroxidase-conjugated goat anti-mouse antibody (1:2000 dilution; Zymed). Reacting bands were visualized by the enhanced chemiluminescence (ECL) Plus detection system (Amersham Biosciences)”.

2.3 RESULTS

2.3.1 cDNA Identification

“A cDNA encoding an open reading frame with high similarity to human cytoplasmic C₁-THF synthase was cloned from human uterine RNA by the German Genome Project (RZPD; GenBankTM/EBI accession number

AL117452). The homology extends the length of the proteins, suggesting that the cDNA encodes another trifunctional C₁-THF synthase (Figure 2.4). This cDNA encodes 917 amino acids plus 390 nt of 3'-noncoding sequence and a poly(A) tail, but lacks a start codon, suggesting that it is truncated at the 5'-end. Blasting this sequence against the Human Genome Database (NCBI Protein Database) revealed the corresponding gene on chromosome 6 at 6q25.2. This gene spans 236 kilobase pairs and encodes the entire cDNA sequence in 27 exons plus an additional 5'-exon that encodes 60 additional N-terminal amino acids. The predicted initiator codon sits within a near-perfect expanded Kozak consensus sequence (Kozak, 1987). The first half of this N-terminal extension has the characteristics of a mitochondrial leader sequence, including the potential to form a positively charged amphipathic α -helix. Truncation of the original cDNA clone was due to the presence of a *NotI* site near the 3'-end of the first exon; *NotI* was used in the cDNA cloning procedure (Wiemann *et al.*, 2001). Subsequently, the RIKEN Mouse Gene Encyclopedia Project (Kawai *et al.*, 2001) identified a full-length mouse cDNA (ID22289) that predicts a protein with 88% identity to the human protein, including the N-terminal extension (Figure 2.4). The mouse cDNA lacks the *NotI* site that caused truncation of the human cDNA. These data suggest that the gene on human chromosome 6 encodes a mitochondrial C₁-THF synthase. Attempts to construct a full-length cDNA by RACE using human uterine RNA were

unsuccessful, probably due to the extremely high GC content (>80%) of the first exon”.

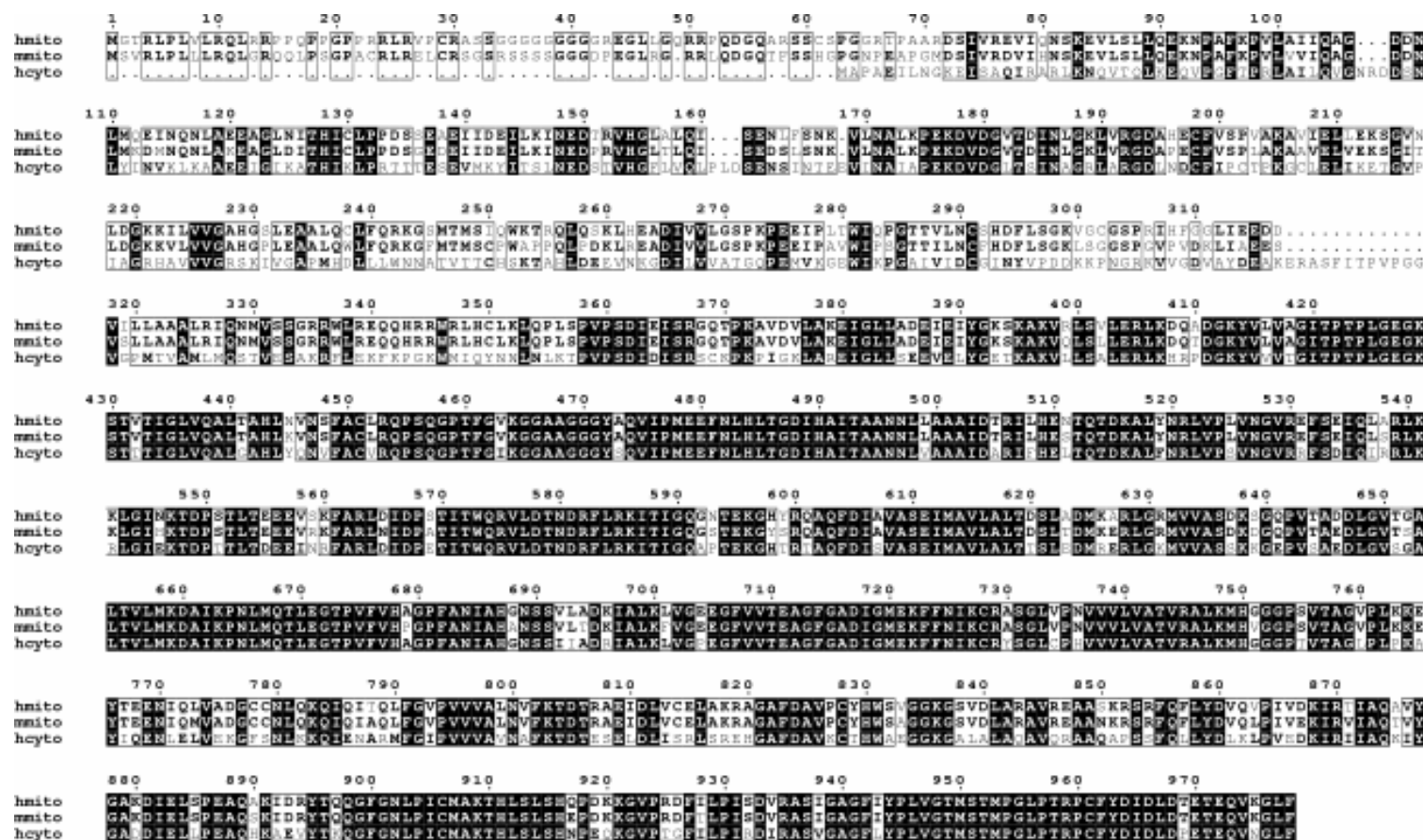


Figure 2.4 Alignment of Human and Mouse Mitochondrial C₁-THF Synthases with Human Cytoplasmic C₁-THF Synthase. *Black boxes* denote identity, and *white boxes* denote conservative substitutions or identities in two of three proteins. The alignment was produced by the INRA server at the Laboratoire de Génétique Cellulaire (available at prodes.toulouse.inra.fr/multalin/multalin.html) using the MultAlin algorithm (Corpet, 1988), and the output was generated by the ESPript program at the same site. *hmito*, human mitochondrial C₁-THF synthase; *mmito*, mouse mitochondrial C₁-THF synthase; *hcyto*, human cytoplasmic C₁-THF synthase. This figure has been published as figure 2 in Prasannan, P. *et al* (2003) *J. Biol. Chem.* **278**, 43178-43187.

2.3.2 PCR amplification of Exon 1 and Partial cDNA

To construct the full-length cDNA, exon 1 (230 bp) and the remaining partial cDNA (2719 bp) were PCR amplified separately from a pGEM-T Easy genomic subclone and a partial cDNA clone, respectively. *Tfl* polymerase was used to amplify exon 1 due to its high GC content (> 80%). The two PCR products were gel-purified separately.

2.3.3 SOE-PCR & TOPO Cloning

PCR-amplified exon 1 was spliced to the remaining cDNA by SOE-PCR to construct a full-length cDNA encoding the human protein (Figure 2.5). The full-length cDNA was gel-purified and cloned into pcDNA3.1D/V5-His-TOPO by directional TOPO cloning. Sequence analysis of the cDNA clone, pcDNA3.1-humito, revealed a base substitution that was probably introduced during PCR. This mutation was repaired using the QuikChange site-directed mutagenesis kit and the full-length cDNA clone was sequenced to confirm the correct sequence (GenBankTM/EBI accession number AY374130).



Figure 2.5 Agarose Gel of Full-length cDNA generated by SOE-PCR. Lane 2 contains spliced full-length cDNA and lane 3 contains partial cDNA. Lane 1 contains 1 kbp DNA ladder. Size of DNA is shown in bp. DNA was visualized by ethidium bromide staining.

2.3.4 Gene Structure

“The human gene encoding C₁-THF synthase spans 236 kilobase pairs on chromosome 6 (Figure 2.6). The coding sequence consists of 28 exons and is interrupted by 27 introns ranging from 89 to 55,350 bp in length. The start codon is present in the first exon, and the 5'-end of exon 1 extends 107 bp

upstream of the ATG start codon (see Chapter 3). The stop codon is present in exon 27, and exon 28 encodes 360 nt of 3'-untranslated region, including a polyadenylation signal (AATAAA). Exon 1 is very GC-rich (>80% GC), containing a CpG island and a *NotI* restriction enzyme site (GCGGCCGC). All of the intron/exon splice sites follow the GT/AG rule (Breathnach and Champon, 1981), except after the terminal exons, 8a and 28 (Table 2.1). A scan of the 5'-flanking sequences by the TESS Web server (available at www.cbil.upenn.edu/tess) using the TRANSFAC Version 4.0 Database predicts numerous potential transcription factor-binding sites, including Sp1, retinoic acid receptor- α 1, and CAAT/enhancer-binding protein- α . The 5'-flanking sequence contains a TATAAA sequence at position –985’.



Figure 2.6 Intron/exon Structure of the Human Mitochondrial C₁-THF Synthase Gene. Exons are shown as *numbered black bars*, and introns as *thin horizontal lines*. Exon and intron sizes and positions are drawn to scale, with the exception of intron 26, which is 55,350 bp. The entire gene spans 236 kb. The actual sizes of each exon and intron are listed in Table 2.1. This figure has been published as figure 4 in Prasannan, P. *et al* (2003) *J. Biol. Chem.* **278**, 43178-43187.

Table 2.1 Intron/exon Splice Junctions of the Human Mitochondrial C₁-THF Synthase Gene. ^aIntrons are numbered starting with intron 1 between exon 1 and 2. ^bThe 5'-end of exon one, and therefore its length, is based on the longest 5'-RACE clone isolated (see Chapter 3). ^cIn one 3'-RACE clone, exon 8a extended an additional 23 bp, for a total length of 162 bp (see Chapter 4). This table has been published as table I in Prasannan, P. et al (2003) *J. Biol. Chem.* **278**, 43178-43187.

Exon no.	Exon size (bp)	Intron sequence	Exon sequence <i>5'-junction 3'-junction</i>	Intron sequence	Intron size (bp)	Intron no. ^a
1	356 ^b		TCCTTC-CGTCAG	gtgagtgtc	9860	1
2	85	tcaatgtag	AGAAGT-ATCCAG	gtaagccga	1460	2
3	51	cttcctaag	GCAGGT-GAGGAG	gtgaggact	89	3
4	54	cttttcag	GCTGGT-GCCGAG	gtaataatg	4936	4
5	125	tctcttag	ATTATA-GGATGG	gtaagaaaa	2748	5
6	101	ctgattcag	AGTAAC-AATCAG	gtaggatgc	2108	6
7	137	tttggttag	GTGTCA-AGCAAG	gtaaatttc	10,879	7
8a ^c	139	cttttcag	ACGGAG-TCCCAG	aatggtgct	6652	8a
8	112	tccttttag	CTTCAC-TGTCAG	gtaaattgc	12,816	8
9	92	tttttacag	GCAAGG-ATTCAG	gtttgttca	3537	9
10	98	ctgctgaag	AACATG-GCCAAG	gtaacactg	3820	10
11	174	tcccctcag	TGACAT-TGCTGG	gtaagacac	10,509	11
12	137	cgaactcag	GATCAC-TGAAAG	gtactgtct	1759	12
13	47	gttggttag	GAGGAG-GAGGAG	gtaagacct	5741	13
14	108	tttctctag	TTCAAC-GATAAG	gtaagaagg	874	14
15	75	tgtttttag	GCTCTG-CTAAAA	gtaagttc	3490	15
16	103	aatcattag	AAACTG-AGAGAG	gtgggtgct	6862	16
17	77	ctctttcag	TATTGG-CGGCAG	gtaggtggt	4204	17
18	141	ctgggtcag	GCGCAG-GATTTG	gtaagtgtt	4554	18
19	69	ctcctgtag	GGGGTG-CTGGAA	gtaagtgtt	6910	19
20	112	cttctctag	GGGACA-TTGTAG	gtaagtat	37,900	20
21	140	tccccacag	TGACCG-CCAAGT	gtaagtgcc	3821	21
22	42	ttatttcag	GTAACG-GAGGAG	gtaagagga	1060	22
23	101	tttttacag	AACATC-CTTCAA	gtaagtcca	536	23
24	178	cttcactag	GACCGA-GTTCAG	gtaagatct	18,800	24
25	108	tttctccag	GTTCCA-CAACAG	gtaaaagtt	2365	25
26	153	ttctcacag	GGTTTT-GGAACG	gtgagtgag	55,350	26
27	121	ctccccag	ATGAGC-ATGCAG	gtaggctga	8939	27
28	360	atttttcag	ACTCCT-AAAACC	aaccagcaa		

2.3.5 CHO Cell Expression and Subcellular Localization

“To determine whether the protein encoded by this cDNA is, in fact, mitochondrial, we expressed the cDNA in CHO cells. The full-length cDNA was cloned into the mammalian expression vector pcDNA3.1D/V5-His-TOPO. This construct fused the 14-amino acid V5 epitope and a His₆ tag to the C terminus of the 2934-bp coding region. Expression of the insert in mammalian cells is driven by the cytomegalovirus promoter. The resulting plasmid, pcDNA3.1-humito, was transfected into CHO cells, and G418-resistant colonies were selected and grown. The cytosolic and mitochondrial fractions from transfected and untransfected (control) CHO cells were isolated. Each fraction was assayed for the mitochondrial marker enzyme glutamate dehydrogenase and the cytoplasmic marker enzyme lactate dehydrogenase. Glutamate dehydrogenase activity ranged from 68 to 95 $\mu\text{mol}/\text{min}/\text{mg}$ of protein in the mitochondrial fractions, compared with 2.4–4 $\mu\text{mol}/\text{min}/\text{mg}$ of protein in the cytoplasmic fractions. The lactate dehydrogenase activity of the mitochondrial fraction was only one-seventh that of the cytoplasmic fraction. These subcellular fractions were then subjected to SDS-PAGE and immunoblotting using antibodies against the V5 epitope (Figure 2.7). A clear signal at ~ 107 kDa was detected in the mitochondrial fraction of the transfected CHO cell line (*lane 2*), but not in the

cytoplasmic fraction (*lane 1*). This mobility was consistent with the expected size of the epitope-tagged construct (~1000 amino acids). No signal was seen in either fraction of the untransfected CHO cell line (*lanes 3 and 4*). These results confirm that this cDNA encodes a protein that localizes exclusively to mitochondria in a mammalian cell line”.

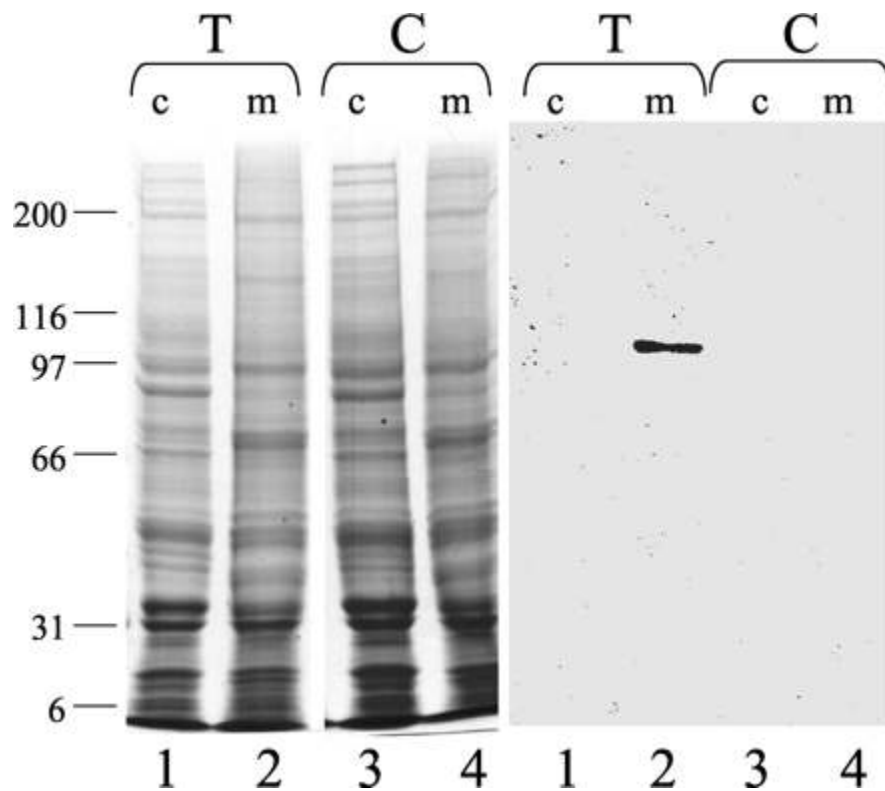


Figure 2.7 Subcellular Localization of Epitope-tagged Human Mitochondrial C₁-THF Synthase Expressed in CHO cells. Shown are a Coomassie Blue-stained SDS-polyacrylamide gel (*left*) and an immunoblot (*right*) of cytoplasmic (*c*) and mitochondrial (*m*) fractions from either CHO cells transfected with pcDNA3.1-humito (*T*; *lanes 1* and *2*) or an untransfected control (*C*; *lanes 3* and *4*). Each lane contains 80 μ g of total protein. This figure has been published as figure 3 in Prasannan, P. *et al* (2003) *J. Biol. Chem.* **278**, 43178-43187.

2.4 DISCUSSION

“The experiments discussed in this chapter describe the events that led to the identification of human mitochondrial C₁-THF synthase gene and its cDNA cloning. The full-length human cDNA encodes a protein of 978 amino acids, including an N-terminal mitochondrial targeting sequence. When the full-length cDNA was expressed in CHO cells, the targeting sequence directed the protein exclusively to mitochondria (Figure 2.7). Alignment of the deduced amino acid sequence with the human cytoplasmic C₁-THF synthase (935 residues) revealed a 62-residue N-terminal extension in the putative mitochondrial protein (Figure 2.4). PSORT II analysis (available at psort.nibb.ac.jp/form2.html) predicted a mitochondrial targeting sequence with a cleavage site between residues 31 and 32. In fact, this cleavage site was confirmed by another research group (Sugiura *et al.*, 2004). The next 31 residues, before alignment with the cytoplasmic protein begins, include an unusual run of 9 consecutive glycines and several basic residues. A very similar N-terminal extension is predicted for the mouse protein (Figure 2.4). Excluding this N-terminal extension, homology to the human cytoplasmic C₁-THF synthase is quite high (61% identity), and the putative mitochondrial protein appears to possess the same domain structure. In the cytoplasmic protein, the N-terminal dehydrogenase/cyclohydrolase domain is ~300 residues, and the C-terminal synthetase domain is ~700 residues (Hum and

MacKenzie, 1991). The two human proteins share 31% identity in the dehydrogenase/cyclohydrolase domains and 73% identity in the synthetase domains, including conserved active-site residues and the 10-formyl-THF-binding site in the synthetase domain (Kirksey and Appling, 1996). However, the putative mitochondrial proteins from human and mouse lack 12 amino acids near the junction between the two domains (position 318) (Figure 2.4)”.

“The human gene encoding this enzyme has several interesting features. The gene is large, spanning 236 kilobase pairs on chromosome 6 at 6q25.2. The gene contains 29 exons (Table 2.1), including the alternative exon 8a found in the intron between exons 7 and 8 (Figure 2.6). This same intron/exon structure is observed for the mouse homolog found on mouse chromosome 10, except that the alternative exon 8a is absent in the mouse gene. Moreover, the genes for the cytoplasmic C₁-THF synthase from rat, mouse, and human have all been shown to contain 28 exons, with introns in nearly identical positions (Patel *et al.*, 2002; Howard *et al.*, 2003). This suggests that an ancestral C₁-THF synthase gene arose before the divergence of the human and rodent lineages, >75 million years ago (Waterston *et al.*, 2002), and genes encoding the mitochondrial and cytoplasmic isozymes are probably related by a gene duplication event”.

This chapter discussed the identification and characterization of a cDNA encoding a potential human mitochondrial C₁-THF synthase. The gene encoding the

mitochondrial isozyme (236 kbp) is found on human chromosome 6 and consists of 28 exons and 27 introns. The putative mitochondrial isozyme is 61% identical to the cytoplasmic isozyme. An N-terminal mitochondrial targeting sequence followed by an unusual sequence of 31 amino acids is present in the putative enzyme before homology starts with the cytoplasmic isozyme. Expression of a full-length cDNA clone in CHO cells showed that the protein encoded was definitely mitochondrial. In fact, expression of the full-length human cDNA in yeast by my colleague Schuyler Pike revealed 10-formyl-THF-synthetase activity, confirming its identification as the human mitochondrial C₁-THF synthase (Prasannan *et al.*, 2003). The distribution of the mitochondrial C₁-THF synthase mRNA in human tissues is investigated in the next chapter.

CHAPTER 3

Tissue Distribution of the mRNA and Transcript Mapping of the 5'-end

3.1 OVERVIEW

Once we confirmed that the full-length cDNA encodes a protein that localizes exclusively to the mitochondria of a mammalian cell line, the next obvious question was whether we could detect expression of human mitochondrial C₁-THF synthase gene in adult human tissues. To achieve this goal, Northern analysis was performed on a Northern blot membrane prebound with human poly(A)⁺ mRNA (Ambion Inc) from several tissues. A probe corresponding to the human mitochondrial isozyme revealed two transcripts, one at ~3.6 kb and the other at ~1.1 kb in most human tissues. The size and tissue distribution of the full-length transcript was quite different from the cytoplasmic isozyme. Next, a 5' Rapid Amplification of cDNA Ends (5'-RACE) was performed to map the 5'-end of the transcripts. In brief, human placental total RNA (Ambion Inc.) was used to synthesize cDNA followed by a nested PCR using 5'-RACE primers and cDNA specific primers. Sequence analysis of the PCR product suggested that both transcripts from this gene initiate at or near position -107. Portions of this work have been published in the *Journal of Biological Chemistry* in the issue of October 31st, 2003 (Prasannan, P., Pike, S., Peng, K., Shane, B. and Appling, D.R. (2003) *J. Biol. Chem.* **278**, 43178-43187) and these are identifiable by quotation marks and increased indent.

3.2 EXPERIMENTAL PROCEDURES

3.2.1 Northern Analysis

Northern analysis was performed in conjunction with my colleague Schuyler Pike. A FirstChoice Northern human blot I kit was obtained from Ambion Inc, with poly(A)⁺ mRNA from the following adult human tissues: brain, placenta, skeletal muscle, heart, kidney, pancreas, liver, lung, spleen, and thymus. Probes were synthesized by asymmetric PCR using Ambion's Strip-EZTM PCR Kit and [α -³²P]dATP (3000 Ci/mmol, PerkinElmer) according to manufacturer's instructions. The two probes represented the 5'- and 3'-ends of the mitochondrial C₁-THF synthase cDNA. A probe was also synthesized for detection of the human cytoplasmic C₁-THF synthase.

a) PCR Probe Synthesis (Mitochondrial isozyme)

The 5'-end probe was synthesized by asymmetric amplification using 1 ng pcDNA3.1-humito (see Chapter 2) as the template with *Taq* DNA polymerase (Applied Biosystems), and primer GS5'SOE (0.1 μ M; see 2.2.1) for the sense strand and primer GSI (10 μ M; 5'-CCGCTCGAGCAAGGCATTGAGGACTTTGTTGCT-3') for the antisense strand. This 304-bp probe covered nt +215 to +518. (The A of the ATG start codon is designated +1). The 3'-end probe was also synthesized in the same method using the same above template and polymerase, and primer DRA3 (0.1 μ M; 5'-GATGCAGTCCCCTGCTATCA-3') for the sense strand and primer TOPO3'

(10 μ M; see 2.2.1) for the antisense strand. This 465-bp probe covered nt +2469 to +2933, ending just before the stop codon. 20 μ Ci [α - ^{32} P]dATP was used for both probe syntheses in a volume of 20 μ l.

b) PCR Probe Synthesis (Cytoplasmic isozyme)

“The plasmid pUC13/HS230 (obtained from Dr. R. E. MacKenzie, McGill University), which contains a 230-bp fragment (+2349 to +2580) near the 3'-end of the human cytoplasmic C₁-THF synthase cDNA (Hum *et al.*, 1988), was linearized by digestion with SacI. A linear PCR amplification method (following the Strip-EZTM PCR kit instructions) was used to synthesize the probe. The antisense primer used was 10 μ M -20 primer # 1211 (5'-GTAAAACGACGGCCAGT-3'), which is complementary to the vector sequences flanking the insert”.

c) Determination of Radiolabel Incorporation by TCA Precipitation

This was done to determine the number of incorporated counts per minute (cpm) of the PCR reaction as per Ambion's Strp-EZTM PCR kit protocol. 150 μ l of salmon sperm DNA (1mg/ml) was sheared by heating at 100°C and transferred into a microfuge tube containing no nucleases. To that, 2 μ l of the PCR reaction was added, and mixed well. After adding aqueous scintillation fluid, the total radiolabel in 50 μ l of the mixture was measured in a scintillation counter. 50 μ l of the above mixture was also mixed with 2 ml of cold 10% trichloroacetic acid (TCA) in a glass tube. The nucleic acids were precipitated by incubating on ice for 10 min and the precipitate

was filtered through a Whatman GF/C glass fiber filter. After washing the tubes with 10% TCA (2 X, 1 ml each) and 95% ethanol (1 X, 3ml), the washes were filtered as well. The filter was kept in a scintillation vial containing aqueous scintillation fluid, and the incorporated radiolabel was measured in a scintillation counter. The cpm obtained was multiplied by 1.5 to give the cpm/ μ l of probe. The cpm of incorporated label, when divided by the cpm of total radiolabel (unincorporated & incorporated) gave the fraction of label incorporated, which when multiplied by 100 gave percent incorporation.

d) Blot Hybridization & Phosphorimaging

A Northern blot membrane prebound with human poly(A)⁺ mRNA from several tissues was prehybridized in 10 ml of Ambion's ULTRAhybTM Ultrasensitive Hybridization Buffer at 42°C for 1 h. The buffer was removed and 10 ml hybridization buffer containing the probe (1×10^6 cpm/ml of hybridization solution) was added, and left to hybridize overnight at 42°C in hybridization buffer in a roller bottle. The membrane was then washed twice with NorthernMax low stringency wash solution (Ambion Inc.) for 10 min each at 42°C, and twice with NorthernMax high stringency wash solution (Ambion Inc) for 30 min each at 42°C. The membrane was covered in saran wrap, exposed overnight to a storage phosphor screen (Amersham Biosciences), and imaged using an Amersham Biosciences 445 SI PhosphorImager.

e) Probe degradation & Removal

The probe was removed immediately after each use by stripping the blot using reagents supplied in the kit. Briefly, the blot was incubated in 1X probe degradation buffer (Ambion Inc.) for 2 min at room temperature, and then for 10 min at 68°C. The blot was then incubated in 1X blot reconstitution buffer (Ambion Inc.) containing 0.1% SDS for 10 min at 68°C. The blot was examined for residual probe by exposing the membrane to a storage phosphor screen. Any residual signal was removed by 0.1% SDS wash for 10 min at 68°C before the next use.

3.2.2 5' RNA Ligase Mediated Rapid Amplification of cDNA Ends

(5' RLM-RACE)

5'-RACE was performed to map the 5'-end of the transcript using human placental total RNA (Ambion Inc.) and the FirstChoice RLM-RACE kit (Ambion Inc.) as per manufacturer's instructions.

a) Primer Design

“Nested antisense primers specific to the cDNA were designed for use with the two nested 5'-RACE primers provided in the kit (see Figure 3.2). The cDNA-specific inner primer (GSI2, 5'-CGCCTCGAGACGGCTGGTTCTCAGGGGACAC-3', with the *Xho*I site underlined) was complementary to nt -9 to -30 in the 5'-untranslated region. The cDNA-specific outer primer (GSO2, 5'-

AGCGCGACAGGGCACACGGAG-3') was complementary to nt +93 to +73.

The primers were 80 bp apart that enabled distinguishing between the nested PCR products based on their size. The 5'-RACE inner primer and the cDNA-specific inner primer had *Bam*HI and *Xho*I sites, respectively, at their 5'-ends to facilitate cloning”.

b) RNA Processing

10 µg of human placental total RNA was treated with calf intestinal phosphatase (CIP) as described in Appendix IV. Acid phenol was prepared by equilibrating phenol with acetate buffer (pH 5). The CIP reaction was terminated by adding a mixture of ammonium acetate (15 µl), ddH₂O (115 µl), and acid phenol:chloroform:isoamylalcohol (150 µl; in a ratio of 25:24:1) and vortexing thoroughly. The mixture was centrifuged at 10,000 X g for 5 min. at room temperature and the top aqueous phase was transferred into another tube. The aqueous phase was mixed with 150 µl chloroform and centrifuged again at 10,000 X g for 5 min. at room temperature. The top aqueous phase was again transferred into another tube and mixed with 150 µl isopropanol. The mixture was incubated on ice for 10 min and centrifuged at 10,000 X g for 20 min. Isopropanol was removed carefully, the pellet (brown in color) rinsed with 0.5 ml cold 70% ethanol, and centrifuged again at 10,000 X g for 5 min. Ethanol was removed, the pellet air-dried, and resuspended in 11 µl nuclease-free water. The CIP-treated RNA was incubated with Tobacco Acid Pyrophosphatase (TAP) followed by RNA adapter ligation as

described in Appendix IV. 1 µl of CIP-treated RNA was used for ‘minus-TAP’ control reaction.

c) Reverse Transcription

The ligated RNA or ‘minus-TAP’ control was reverse transcribed as described in Appendix IV.

d) Nested PCR (Appendix IV)

An “outer” PCR was performed with the reverse transcriptase (RT) reaction as the template, primers GSO2 (cDNA-specific outer primer) and 5’-RACE outer primer, and *Tfl* DNA polymerase. An “inner” PCR was done with the outer PCR product as the template, primers GSI2 (cDNA specific inner primer) and 5’-RACE inner primer, and *Tfl* DNA polymerase. The products from both “outer” and “inner” PCRs were analyzed on a 2% agarose gel.

“PCR fragments generated in the “inner” PCR of 5'-RACE was gel-purified using QIAquick gel extraction kit, digested with *Bam*HI and *Xho*I, and ligated into *Bam*HI/*Xho*I-digested pBluescript II KS(+/-) vector (Stratagene). The ligation reaction was transformed into RbCl-competent *E. coli* XL1-Blue cells, and positive colonies were selected on YT/ampicillin plates. Colonies were screened by PCR using T7 reverse (5'-GTAATACGACTCACTATAGGGC-3') and T3 forward (5'-AATTAACCCTCACTAAAGGG-3') vector primers, and plasmids were prepared by QIAprep Spin Miniprep kit for sequence analysis”.

3.3 RESULTS

3.3.1 Northern Analysis

“A Northern blot membrane prebound with human poly(A)⁺ RNA from several tissues was obtained from Ambion Inc. A 304-bp 5'-end probe spanning nt 215–518 of the human mitochondrial C₁-THF synthase cDNA revealed two bands: one at ≈3.6 kb and the other at ≈1.1 kb (Figure 3.1A). The upper band corresponded to the expected size of the full-length transcript. To ensure that the 1.1-kb band was not an artifact, we washed the membrane for an additional 30 min with high stringency wash buffer at 50 °C. The additional wash did not eliminate either band. The upper and lower band distributions were very similar, with the highest transcript levels being in placenta, thymus, and brain. Expression was low in liver and skeletal muscle and barely detectable in heart”.

“To determine the relationship of the 3.6- and 1.1-kb transcripts, a 465-bp probe was synthesized that ended just before the stop codon. This 3'-probe detected only the 3.6-kb transcript (Figure 3.1B), suggesting that the 1.1-kb transcript represents just the 5'-end of the cDNA”.

“We also compared the tissue distribution of the mitochondrial C₁-THF synthase transcript with that of the cytoplasmic isozyme. Using a 230-bp probe from the 3'-end of the cytoplasmic C₁-THF synthase cDNA (Hum *et*

al., 1988), a 3.3-kb transcript was observed (Figure 3.1C). The tissue distribution of this transcript differed from that of the mitochondrial isozyme, being highest in liver, kidney, and skeletal muscle. Thus, the human mitochondrial and cytoplasmic C₁-THF synthase isozymes are encoded by distinct transcripts that do not cross-hybridize with these probes under the wash conditions used”.

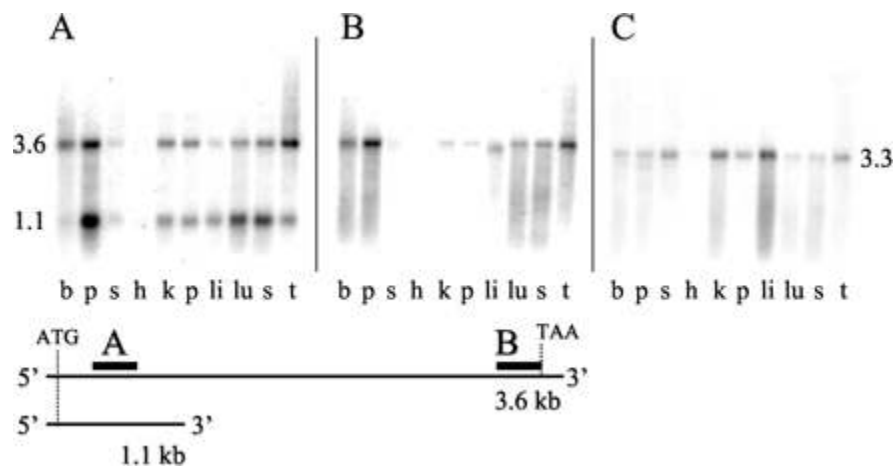


Figure 3.1 Northern Blot Analysis of Mitochondrial C₁-THF Synthase Transcripts in Adult Human Tissues. A human multiple-tissue RNA blot was hybridized with ³²P-labeled probes to the 5'-end (A) or 3'-end (B) of human mitochondrial C₁-THF synthase cDNA. In C, the membrane was hybridized with a probe from the 3'-end of the human cytoplasmic C₁-THF synthase cDNA. The lanes in each panel contain RNA from (left to right) brain (b), placenta (p), skeletal muscle (s), heart (h), kidney (k), pancreas (p), liver (li), lung (lu), spleen (s), and thymus (t). The schematic diagram below shows the relative locations of the probes used for A and B to obtain the 3.6- and 1.1-kb transcripts. This figure has been published as figure 5 in Prasanna, P. *et al* (2003) *J. Biol. Chem.* **278**, 43178-43187.

3.3.2 Transcript Mapping

“A 5'-RACE experiment was done to determine the transcriptional start site(s). 5'-RACE was performed as described under 3.2.2 using 10 µg of human placental total RNA for first-strand cDNA synthesis by reverse

transcription. This was followed by a first round of PCR (“outer” PCR), which gave no detectable specific product. Two μ l of the outer PCR product were used in a second round of PCR with nested primers (“inner” PCR), yielding a specific product of <300 bp. The final PCR product was gel-purified and subcloned. Nine colonies were screened by PCR, and all of them gave a product of between 220 and 298 bp. Three of the nine clones were sequenced, and all of them exhibited the same 5'-end 107 bp upstream of the ATG start codon (Figure 3.2). These results suggest that the majority of the transcripts from this gene initiate at or near position –107, and it appears that both the 3.6- and 1.1-kb transcripts initiate from this site”.

3.4 DISCUSSION

“Expression of the gene was detected in most human tissues, but transcripts were highest in placenta, thymus, and brain. Expression was low in liver and skeletal muscle and barely detectable in heart. A mouse cDNA has also been identified that predicts a protein with 88% identity to the human protein, including the N-terminal extension, suggesting that this mitochondrial C₁-THF synthase will be found in all mammals”.

“The tissue distribution of the mitochondrial C₁-THF synthase is quite different from that of the cytoplasmic isozyme (Figure 3.1). Whereas the cytoplasmic transcript is most abundant in liver and kidney, the transcripts for the mitochondrial isozyme are relatively low in those tissues, but highest in placenta, followed by thymus, spleen, brain, and lung. The low expression of the mitochondrial isozyme in liver probably contributed to difficulties experienced by earlier members of the lab in purifying the protein from liver mitochondria. Although the ratio of the two transcripts varies somewhat from tissue to tissue, both are present in every tissue assayed, even heart (Figure 3.1). The short transcript is significantly reduced in brain”.

“Using RNA from human placenta, a single 5'-transcriptional start site at position –107 was identified by 5'-RACE (Figure 3.2). It appears that both the 3.6- and 1.1-kb transcripts initiate from this site because only a single 5'-end was identified. A BLAST search of the Human EST Database with the 5'-

end of the human cDNA revealed >100 entries. Four ESTs extended beyond position -107 (Figure 3.2). BG481636 (position -276) and BE735249 (position -268) were isolated from choriocarcinoma mRNA; BQ062382 (position -119); and BQ055629 (position -118) were isolated from a lymphoma cell line. Thus, it appears there may be some heterogeneity in the 5'-transcriptional start site, depending on the tissue or cell type”.

According to Northern blot analysis, the mitochondrial C₁-THF synthase gene was expressed in most of the human tissues and the transcript was quite different from that of the cytoplasmic isozyme. A short transcript of ~1.1 kb was also revealed together with the full-length transcript. The short transcript was observed only when the Northern blot was done using the 5' probe, suggesting that it represented just the 5'-end of the cDNA. A 3'-RACE experiment was next performed to map the 3'-end of the short transcript.

CHAPTER 4

Mapping of the 3'-end of the Short Transcript and cDNA Cloning, Expression, and Purification of the Short Isoform of Human Mitochondrial C₁-THF Synthase

4.1 OVERVIEW

Northern analysis of human mitochondrial C₁-THF synthase detected the 3.6 kb full-length transcript along with a 1.1 kb short transcript (see Chapter 3, Figure 3.1A). The objectives of the experiments described here were to map the 3'-end of this short transcript, and to examine the possibility of the short transcript encoding a functional protein. First, mapping of the short transcript was performed by RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) using the FirstChoiceTM RLM-RACE kit from Ambion Inc. In brief, human placental total RNA (Ambion Inc.) was used to synthesize cDNA by reverse transcription followed by a nested PCR using 3'-RACE primers and cDNA specific primers. Sequence analysis of the PCR product showed that the short transcript arose as a result of alternative splicing. To achieve the second goal, the short isoform cDNA was PCR-amplified from pcDNA3.1-humito using custom-designed primers, and cloned into an *E. coli* expression vector. The recombinant protein was expressed in RbCl-competent Rosetta 2(DE3)pLysS *E. coli* strain (Novagen) and purified. No dehydrogenase activity was found associated with the purified short isoform. Nevertheless, the

purified protein was successfully used to raise polyclonal antibodies in rabbits. Portions of this work have been published in the Journal of Biological Chemistry in the issue of October 31st, 2003 (Prasannan, P., Pike, S., Peng, K., Shane, B. and Appling, D.R. (2003) *J. Biol. Chem.* **278**, 43178-43187) and these are identifiable by quotation marks and increased indent.

4.2 EXPERIMENTAL PROCEDURES

4.2.1 3' RNA Ligase Mediated Rapid Amplification of cDNA Ends

(3' RLM-RACE)

For mapping the 3'-end of the 1.1-kb transcript, first-strand cDNA was synthesized from human placental total RNA by reverse transcription as described in Appendix IV. Nested sense primers specific to the cDNA were designed for use with the two nested 3'-RACE primers. The cDNA-specific inner primer (3'-RACE GSI, 5'-CGCCTCGAGGA^{ACTTGT}TTAGCAACAAAGTCCT-3', with the *XhoI* site underlined) was equivalent to nt +485 to +508. The cDNA-specific outer primer (3'-RACE GSO, 5'-CGCCTCGAGCTCCCTCCAGATAGCAGTGAA-3') was equivalent to nt +390 to +410. The 3'-RACE inner primer and the cDNA-specific inner primer had *Bam*HI and *Xho*I sites, respectively, at their 5'-ends to facilitate cloning. Taq DNA Polymerase was used for nested PCR.

“PCR fragments generated in the “inner” PCR of 3'-RACE was gel-purified, digested with *Bam*HI and *Xho*I, and ligated into *Bam*HI/*Xho*I-digested pBluescript II KS(+/-) vector. The ligation reaction was transformed into RbCl-competent *E. coli* XL1-Blue cells, and positive colonies were selected on YT/ampicillin plates. Colonies were screened by PCR using T7 reverse (5'-GTAATACGACTCACTATAGGGC-3') and T3 forward (5'-AATTAACCCTCACTAAAGGG-3') vector primers, and plasmids were prepared for sequence analysis. This 1.1-kb cDNA has been submitted to the GenBankTM/EBI Data Bank under accession number AY374131”.

4.2.2 cDNA Cloning of Short Isoform of Mitochondrial C₁-THF Synthase

cDNA for the short isoform was PCR-amplified from pcDNA3.1-humito (see Chapter 2) using KOD Hot Start DNA polymerase (Novagen) and primers SHOT5' (5'-CGCCCATATGGGCACGCGTCTGCCG-3'; *Nde*I site underlined), and SHOT3' (5'-CGCCTCGAGGATCACGCGCCTGCACTCCAGCCTGGTGACAGAACGAGACTCCGTCTTGCTTTGAAGCTGGCG-3'; *Xho*I site underlined) containing the 45 extra nucleotides not present in full-length human mitochondrial C₁-THF synthase cDNA. The product (825-bp) was gel-purified using the QIAquick gel extraction kit, double-digested with *Nde*I and *Xho*I, purified using QIAquick PCR purification kit (QIAGEN) and cloned into *Nde*I/*Xho*I digested *E. coli* expression vector pET22b (Novagen). The ligation reaction was transformed into RbCl-competent *E. coli* XL1-

Blue cells and positive colonies were selected on Luria-broth (LB)/agar (1% tryptone, 0.5% yeast extract, 1% NaCl, and 2% agar) plates containing 50µg/ml ampicillin. The colonies were screened by PCR with a vector primer and a gene-specific primer, and positive plasmids were prepared using QIAprep Spin Miniprep kit. The short isoform construct, pET22b-short, was sequenced and the correct sequence was confirmed. Attempts to express the short isoform protein from this construct in *E. coli* were not successful. So, the short isoform cDNA was subcloned into the pMal-c2x H10TEV vector (obtained from Dr. John Tesmer (Kristelly *et al.*, 2004)) using KOD Hot Start DNA polymerase and primers hmcleavedlong5' (5' TATAGGATCCAGCAGCGGCGGCGGAGGC-3'; *Bam*HI site underlined) and short isoform3' (5'-CGCAAGCTTTTAGATCACGCGCCTGCACTC-3'; *Hind*III site and following stop codon underlined). The 5' primer, hmcleavedlong5' was designed to amplify the cDNA from nucleotide +94 onwards only (the A of the ATG start codon of the full-length cDNA is designated +1), thereby eliminating the N-terminal 31 codons, encoding the mitochondrial presequence, from the construct. The PCR product (731-bp) was gel-purified using a QIAquick gel extraction kit, double-digested with *Bam*HI and *Hind*III, purified using QIAquick PCR purification kit, and cloned into *Bam*HI/*Hind*III digested *E. coli* expression vector pMal-c2x H10TEV. The ligation reaction was transformed into RbCl-competent *E. coli* XL1-Blue cells and positive colonies were selected on LB agar plates containing 50µg/ml ampicillin and 30µg/ml chloramphenicol (LB/Amp/Cam). The colonies were screened by PCR

with a vector primer and a gene-specific primer, and plasmids were prepared from positive colonies using QIAprep Spin Miniprep kit. The short isoform construct, pMal-short isoform, was sequenced and the correct sequence was confirmed. A schematic representation of the pMal expression system used for short isoform purification is given in Figure 4.1.

4.2.3 Expression and Purification of Short Isoform

pMal-short isoform was transformed into competent Rosetta 2(DE3)pLysS *E. coli* strain and transformants were selected on LB/Amp/Cam agar plates at 37°C. A single colony was used to inoculate 5ml LB/Amp/Cam liquid medium and grown at 37°C while shaking for ~ 7 hrs. This was used to inoculate a 25 ml LB/Amp/Cam medium that was grown overnight at 37°C while shaking. One liter of LB/Amp/Cam medium supplemented with 0.2% glucose was inoculated with the overnight culture to an initial OD₆₀₀ of 0.1 and grown at 37°C while shaking until an OD₆₀₀ of 0.5. The culture was brought to 15°C, and the expression of the fusion protein (Maltose binding protein/Short isoform; MBP/SI) was induced overnight using 50 µM IPTG at 15°C while shaking. The cells were harvested by centrifugation at 4300 X g for 20 min. and washed using 1X extraction/wash buffer (50 mM sodium phosphate (pH 7.0), 300 mM NaCl, 5 mM 2-mercaptoethanol (2-ME), and 20% glycerol). The cell pellet was resuspended in 20 ml of 1X extraction/wash buffer containing 1 mM

phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine-HCl and two complete-mini EDTA-free protease inhibitor cocktail tablets (Roche). The cells were sonicated with a Vibra cell (Danbury, CT) Model VC40 on ice using a 2 mm probe for 5 X 15 sec. with 30 sec. interruption in between each cycle. The sonicated cell suspension was centrifuged at 30,600 X g for 20 min. at 4°C and the supernatant was the cell extract. Purification of short isoform was done using TALON Cobalt metal affinity resin (BD Biosciences) by a batch/column method. In brief, 10 ml resin was equilibrated twice with 50 ml of the 1X extraction/wash buffer. The equilibrated resin was mixed with the cell extract and gently shaken at 4°C for 20 min. The suspension was centrifuged at 700 X g for 5 min and the supernatant was removed. The resin was washed with 50 ml of 1X extraction/wash buffer by agitating at 4°C for 10 min. The resin was then resuspended in 1X extraction/wash buffer, transferred to a column, allowed to settle by gravity, and washed once again with 50 ml 1X extraction/wash buffer. The column was washed with 50 ml 1X extraction/wash buffer containing 10 mM imidazole. The bound fusion protein, MBP/SI, was eluted using 1X extraction/wash buffer containing 150 mM imidazole and 1 ml fractions were collected. The protein-containing fractions were pooled and protein concentration determined by the Bradford assay using BSA as a standard as given in appendix IV. The fusion protein was cleaved by mixing with TEV protease (see section 4.2.4) at a MBP/SI:TEV protease ratio of 5:1 (w/w) at room temperature while dialyzing against 20 mM Tris-HCl, 5 mM 2-ME, 100 mM KCl, 10% glycerol

(pH 8.0) overnight. The TEV-cleaved and dialyzed protein was then loaded onto a 5 ml Talon Cobalt column and the flow through fractions containing protein (short isoform/SI) were collected and pooled. A Bradford assay was performed to determine the protein concentration. The purified short isoform was subjected to N-terminal sequencing at Austin Protein Sequencing Facility at the University of Texas.

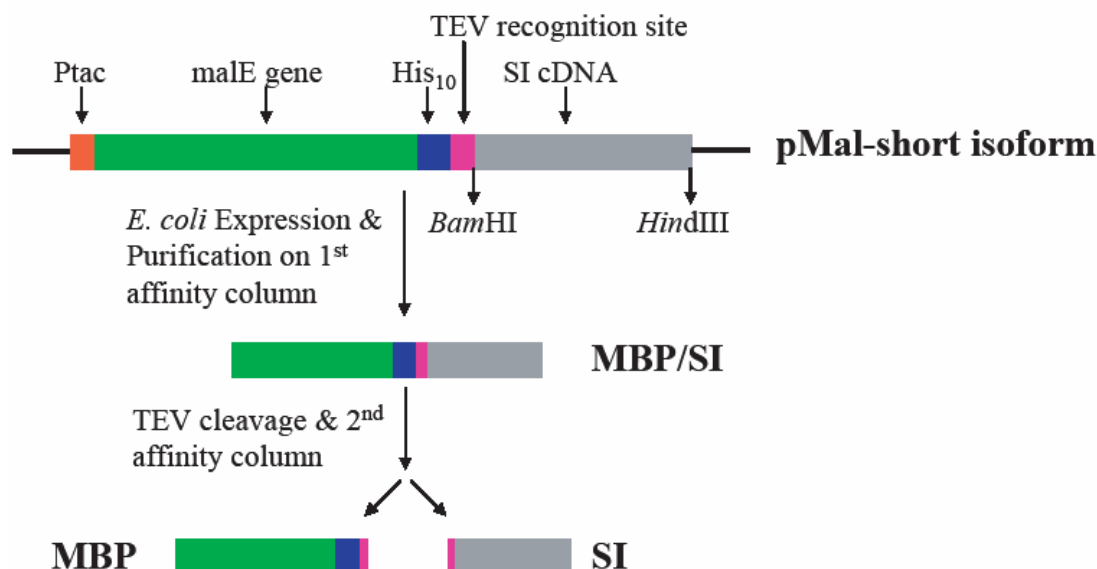


Figure 4.1 Schematic Representation of pMal Expression System for Short Isoform Purification. Description of short isoform cDNA cloning into pMal, *E. coli* expression, and purification are given in sections 4.2.2 and 4.2.3. Abbreviations used are pMal, pMal-c2xH10TEV vector; TEV, tobacco etch virus protease; MBP, maltose binding protein; SI, short isoform of human mitochondrial C₁-THF synthase. Ptac is a hybrid promoter of trp promoter and lacUV5 promoter/operator. malE gene encodes MBP.

4.2.4 Expression and Purification of TEV protease

The plasmid pRK793/TEV (obtained from Dr. John Tesmer (Kristelly *et al.*, 2004) (Kapust *et al.*, 2001) was used for the expression of TEV protease in Rosetta 2(DE3)pLysS *E. coli* strain. The plasmid needs to be freshly transformed into the competent cells for successful overexpression of TEV protease. A 1 liter

LB/Amp/Cam liquid culture was grown at 37°C to an OD₆₀₀ of 0.5 and protein expression was induced with 100 µM IPTG at room temperature overnight. Purification of His-tagged TEV protease was done similar to the short isoform (section 4.2.3) by a batch/column method except for using 5 ml TALON Cobalt metal affinity resin, and the following 1X extraction/wash buffer (25 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5 mM 2-ME, and 10% glycerol). 1X extraction/wash buffer containing 20 mM and 500 mM imidazole were used for the washes and elution, respectively. A Bradford assay was performed on the purified TEV protease. The enzyme was stored in aliquots at -70°C. A 12% SDS-polyacrylamide gel of TEV protease purification is shown in Figure 4.2.

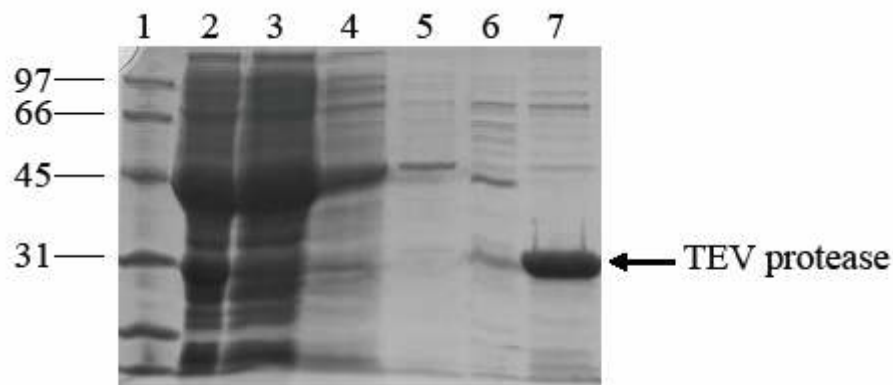


Figure 4.2 Purification of TEV Protease. Shown is a Coomassie Blue-stained SDS-polyacrylamide gel of fractions from TEV protease purification. Molecular weight markers (*lane 1*) Load (*lane 2*), flow-through (*lane 3*), washes (*lanes 4-6*), and TEV protease (29 kDa; *lane 7*). Size of molecular weight markers (kDa) are shown on the left.

4.2.5 Polyclonal Antibody Production

Short isoform was concentrated using a Centriprep YM-10 concentrator (Millipore) by centrifuging at 1750 X g at room temperature, mixed with 6X SDS sample buffer, boiled for 5 min and electrophoresed by SDS-PAGE on a 12% polyacrylamide gel for 60 min at 180 V. The gel was stained, destained and the band containing the short isoform (35 kDa, see Figure 4.6; *lane 3*) was cut out and sent to The University of Texas M.D. Anderson Cancer Center (Department of Veterinary Sciences, Bastrop, TX) for polyclonal antibody production in rabbits. A protocol of antisera production is given in Appendix IV.

4.2.6 Enzyme Assay of Short Isoform

A NADP-dependent methylene-THF dehydrogenase assay (West *et al.*, 1993) was performed on the TEV-cleaved MBP/SI. The 1X extraction/wash buffer used for purification of short isoform for dehydrogenase assay was 20 mM Tris-SO₄ (pH 7.5), 100 mM KCl, 5 mM 2-ME, and 20% glycerol. Briefly, 5,10-methylene-THF was prepared by incubating a mixture of 1 ml THF (Walkup and Appling, 2005) and 1.2 µl 37% formaldehyde in a microfuge tube at 37°C for 10 min. 50 µl TEV cleaved MBP/SI (0.2 µg/µl) after overnight dialysis was mixed with 400 µl reaction mixture (0.05 M HEPES-KOH (pH 7.5), 0.1 M KCl, 0.5 mM NADP). 50 µl 5,10-methylene-THF was added to the mixture and incubated at 37°C for 10 min. The reaction was stopped by adding 0.5 ml of 3% perchloric acid, vortexed, and incubated at room temperature for 5 min to allow for the conversion of 10-formyl-THF to 5,10-methenyl-THF. The mixture was centrifuged at 10,000 X g for 5 min and absorbance of the supernatant was measured at 350 nm. 50 µl of DAY4 (Roje *et al.*, 2002) extract (prepared in 25 mM Tris-SO₄ (pH 7.5), 10 mM KCl, 10 mM 2-ME, 1 mM PMSF extraction buffer) and 50 µl of 1 X extraction/wash buffer were used as the positive and negative controls, respectively.

4.3 RESULTS

4.3.1 Alternative Splicing

“A 3'-RACE experiment was performed to determine the 3'-end of the short 1.1-kb transcript observed on Northern blots (see Chapter 3, Figure 3.1A). One μ g of human placental total RNA was used for first-strand cDNA synthesis. This was followed by a first round of PCR (“outer” PCR), which gave no detectable specific RACE product. One μ l of the outer PCR product was used in a second round of PCR with nested primers (“inner” PCR). Four distinct PCR products of 500, 350, 200, and 100 bp were detectable on a 2% agarose gel (A smear at the top of the gel was also observed, produced from the full-length transcript) (Figure 4.3). Based on the 1.1-kb length of the short transcript and the position of the inner primer, the 500- and 350-bp RACE products were gel-purified and cloned separately. Six of the clones were sequenced to determine the 3'-end of the clones. All of these clones represented the short transcript, in which exon 7 is spliced to a previously unrecognized exon, termed exon 8a, which sits in the intron between exons 7 and 8 (Figure 4.4A). Exon 8a appears to be 139 bp long, although in one clone, the 3'-end extended 162 bp. It contains a stop codon after 45 nucleotides and a polyadenylation signal near its 3'-end. Thus, the 3.6- and 1.1-kb transcripts share the first seven exons and then diverge at exon 8/8a. The 1.1-

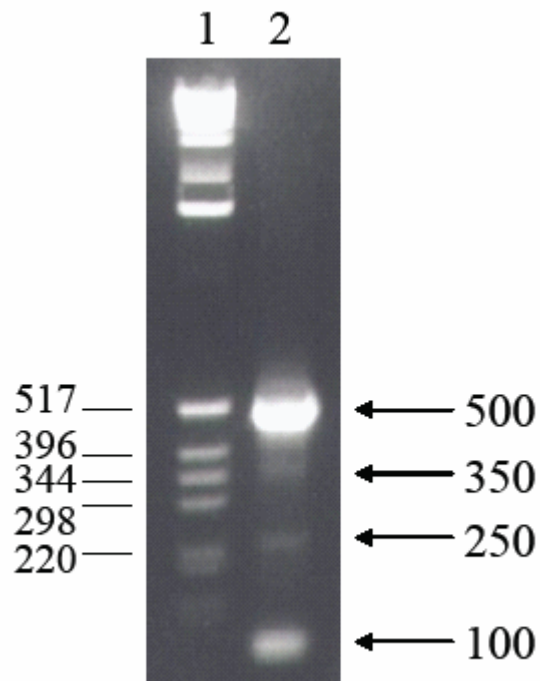


Figure 4.3 Agarose gel of 3' RACE "inner" PCR products. *Lane 2* contains the PCR products and *Lane 1* contains 1 kbp DNA ladder. Size of DNA is shown in bp. DNA was visualized by ethidium bromide staining.

kb transcript would be translated into a 275-amino acid protein in which the first 260 amino acids are identical to the full-length protein, followed by 15 unrelated amino acids (GenBankTM/EBI accession number AY374131) (Figure 4.4, *B* and *C*)".

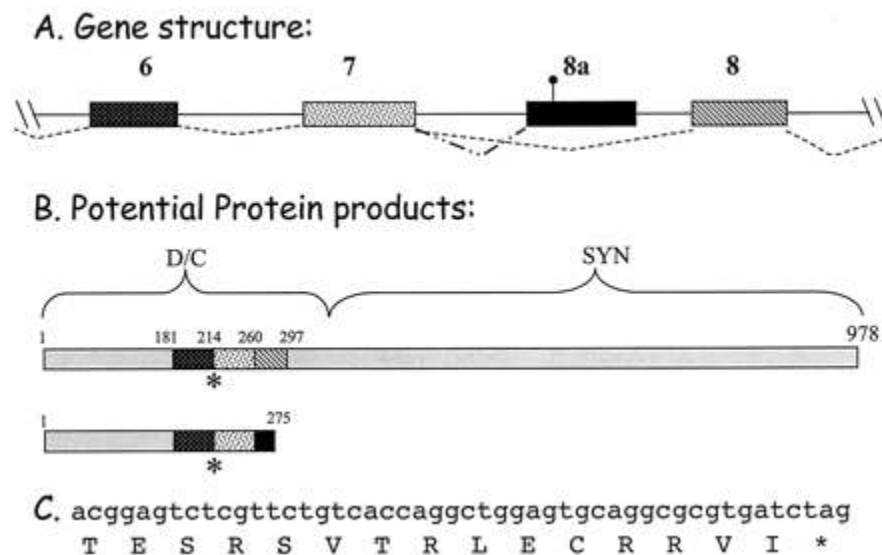


Figure 4.4

Alternative Splicing of the Human Mitochondrial C₁-THF Synthase Transcript. *A*, partial* gene structure and splicing pattern. The alternative exon 8a is in the intron between exons 7 and 8. In the 3.6-kb transcript, exon 7 is spliced to exon 8. In the 1.1-kb transcript, exon 7 is spliced to exon 8a. Exon 8a contains a stop codon (*black dot*) after 15 sense codons and contains a polyadenylation signal (AATAAA) near its 3'-end. There is no homology between exons 8a and 8. *B*, potential protein products. The long transcript is translated into a 978-amino acid protein, whereas the short transcript is translated into a 275-amino acid protein. The amino acid sequences of the two proteins are identical through the first seven exons, encoding 260 amino acids. The short protein has 15 amino acids from exon 8a in place of the amino acids encoded by exon 8. The junction between the dehydrogenase/cyclohydrolase (*D/C*) and synthetase (*SYN*) domains is predicted to lie within amino acids 330–350 (see Figure 2.4). The *asterisks* indicate variable 3'-splice site selection at the exon 6/7 junction (Figure 4.5). *C*, nucleotide and amino acid sequences of the coding sequence of exon 8a. Sequences 3' to the stop codon are not shown. * For complete gene structure see Figure 2.4. This figure has been published as figure 7 in Prasannan, P. *et al* (2003) *J. Biol. Chem.* **278**, 43178–43187.

“An additional variation was observed upon sequencing the 3'-RACE clones. Several of the clones contained an extra codon at position +643, at the junction between exons 6 and 7 (Figure 4.5). This extra valine codon appears to arise from variation in the 3'-splice acceptor site during the splicing of exon 6 to exon 7. The 5'-splice site has the GT consensus sequence as the first 2 nt of the intron. The 3'-splice site has two AG consensus dinucleotides at the 3'-end of the intron. If the first AG dinucleotide is used, exon 7 contains 3 additional nt; if the second is used, these 3 nt are not present in exon 7”.

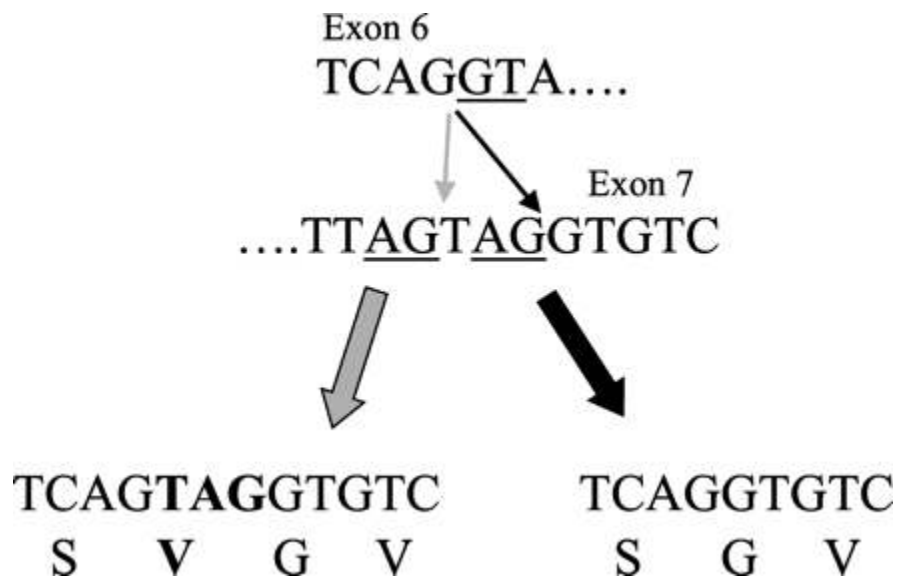


Figure 4.5 Variable 3'-Splice Site Selection at the Exon 6/7 junction. *Upper*, intron/exon junctions for the 3'-end of exon 6 and the 5'-end of exon 7. The first 2 nt of the intron and the alternative AG splice acceptors sites are underlined. *Lower*, alternative splicing products if the first (*left*) or second (*right*) AG acceptor site is used. The amino acid sequence encoded by each product is shown below the nucleotide sequence, and the extra codon and amino acid are in *boldface*. This figure has been published as figure 8 in Prasanna, P. *et al* (2003) *J. Biol. Chem.* **278**, 43178-43187.

4.3.2 cDNA Cloning, Expression, and Purification of Short Isoform

Northern blot analysis of mitochondrial C₁-THF synthase in human tissues revealed a full-length 3.6 kb transcript, and an alternatively spliced shorter transcript of 1.1 kb (Figure 3.1A). If translated, this short transcript would encode a 275-amino

acid protein in which the first 260 amino acids are identical to the full-length protein, followed by 15 residues derived from an exonized Alu element. The pMal-short isoform construct lacks the N-terminal 93 nts encoding the mitochondrial presequence. pMal-c2x H10TEV vector (Kristelly *et al.*, 2004) allowed the expression of the protein of interest as a fusion protein with an N-terminal maltose binding protein (MBP), thereby enhancing its solubility (Fox and Waugh, 2003). This vector includes a His₁₀ tag at the C-terminus of the MBP followed by a TEV protease recognition site and then the multiple cloning site. This fusion protein (MBP/SI; 80 kDa) was expressed in *E. coli* (Figure 4.6; *lane 1*), purified on a TALON Cobalt metal affinity resin that has a low affinity for *E. coli* proteins, and cleaved using TEV protease (29 kDa; recognition sequence is ENLYFQQ with the cleavage site underlined). A second affinity column was used to separate MBP retaining the His₁₀ tag (45 kDa) from SI (35 kDa) in the flow-through. An overnight induction at a low temperature enabled high level expression of MBP/SI. Approximately 5 mg and 500 µg of MBP/SI and SI, respectively, were obtained from a 1 L culture. TEV protease cleavage of MBP/SI was incomplete even at a MBP/SI:TEV protease ratio of 5:1 (Figure 4.6; *lane 2*). In addition, some of the uncleaved MBP/SI failed to bind to the second affinity column and appeared with the short isoform flow-through (Figure 4.6; *lane 3*). Nevertheless, the predominant product was the cleaved short isoform, which remained stable and soluble. On SDS-polyacrylamide gel, SI migrated more slowly than expected for its size (30 kDa). N-

terminal sequencing of SI gave the sequence **GEFGSSSGGGG**, of which the amino acids shown in bold were obtained from the vector after TEV cleavage and the amino acid shown in italic is the 32nd residue of the full-length gene product (1st residue of SI). As mentioned above, the N-terminal 93 nt, which encode the mitochondrial presequence (31 amino acids), were omitted in the pMal-short isoform construct. No methylene-THF dehydrogenase activity was detected in the TEV cleaved MBP/SI.

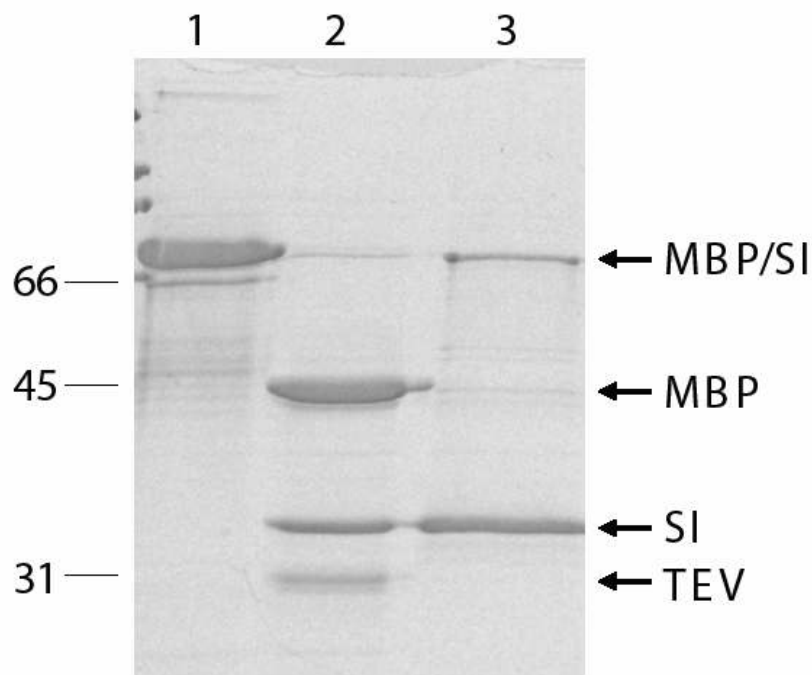


Figure 4.6 Purification of the Short Isoform of Human Mitochondrial C₁-THF Synthase. Shown is a Coomassie Blue-stained SDS-polyacrylamide gel of the fusion protein eluted from the first cobalt column (*lane 1*), products after TEV protease cleavage reaction (*lane 2*), and purified short isoform after second cobalt column (*lane 3*). MBP/SI, maltose binding protein/short isoform fusion protein; MBP, maltose binding protein; SI, short isoform; TEV, Tobacco etch virus protease. Locations and size (kDa) of molecular weight markers are shown on the left.

4.3.3 Specificity of Anti-mito-C₁-THF Synthase Antibodies

Polyclonal antibodies raised against rat cytoplasmic C₁-THF synthase (Cheek and Appling, 1989) cross-react with human mitochondrial C₁-THF synthase (data not shown). Since the human cytoplasmic and mitochondrial proteins share only 31%

identity in the N-terminal dehydrogenase/cyclohydrolase domain (Figure 2.4), we predicted that polyclonal antibodies raised against the short isoform, which represents just the N-terminus of the mitochondrial isozyme, might specifically recognize the mitochondrial isozyme. The preparation of pure short isoform protein, devoid of uncleaved fusion protein, for use as antigen is described under section 4.2.5. Antisera obtained (anti-mito-C₁-THF synthase antibodies) from M.D. Anderson Cancer Center were stored in aliquots at -70°C until use.

To analyze the specificity of the antibodies obtained, purified short isoform of mitochondrial C₁-THF synthase (Figure 4.6, *lane 3*) was electrophoresed on a 7.5% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. The membrane was probed with either anti-mito-C₁-THF synthase antibodies or the pre-immune serum (Figure 4.7; *Panel A*). While the anti-mito-C₁-THF synthase antibodies gave a signal at the size of the short isoform (27 kDa) (*lane 1*), the pre-immune serum gave no signal at the corresponding size (*lane 2*), confirming this antiserum is specific for the antigen (short isoform) used. The anti-mito-C₁-THF synthase antibodies also detected a signal at 72 kDa corresponding to the fusion protein (MBP/SI) (*lane 1*), since the purified protein contained some uncleaved MBP/SI. A protein at 45 kDa, similar to the size of MBP, was seen in immunoblots with the anti-mito-C₁-THF synthase antibodies (*lane 1*) as well as the pre-immune serum (*lane 2*).

To determine whether the polyclonal antibodies raised against the purified short isoform cross-react with the cytoplasmic isozyme, 50 µg of rat liver cytosol or rat spleen cytosol were fractionated on a 7.5% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. The membrane was immunoblotted with either anti-mito C₁-THF synthase or anti-cyto-C₁-THF synthase antibodies (Figure 4.7). While the anti-cyto-C₁-THF synthase antibodies gave a clear signal at the size of the cytoplasmic isozyme (> 100 kDa) in both liver and spleen cytosols (Panel B; *lanes 1 & 2, respectively*), the anti-mito-C₁-THF synthase antibodies gave a faint signal of the same size in rat liver cytosol (Panel C; *lane 1*). No similar signal was observed in rat spleen cytosol (Panel C; *lane 2*). Rat spleen mitochondria, which gave an intense signal (Panel C; *lane 3*) at the size of the mitochondrial isozyme (~ 100 kDa) with the anti-mito-C₁-THF synthase antibodies, were used as a control.

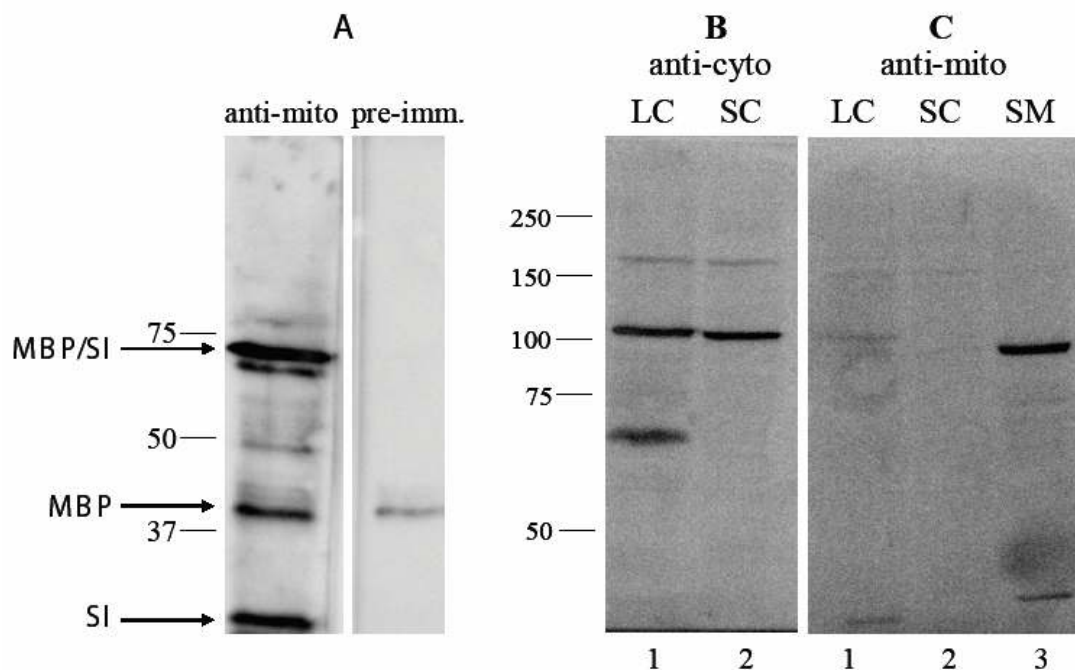


Figure 4.7 Specificity of Anti-mito-C₁-THF Synthase Antibodies. *Panel A.* Immunoblot of purified short isoform (SI) with a 1:1000 dilution of anti-mito-C₁-THF antibodies (anti-mito) (lane 1) or a 1:1000 dilution of the pre-immune serum (lane 2). Each lane contains 1 µg of purified SI. MBP/SI, maltose binding protein/short isoform fusion protein; MBP, maltose binding protein; SI, short isoform. *Panel B.* Immunoblot of cytoplasmic fractions from rat liver (LC; lane 1) and rat spleen (SC; lane 2) with a 1:2500 dilution of anti-cyto-C₁-THF synthase antibodies (anti-cyto, Cheek and Appling, 1989). *Panel C.* Immunoblot of cytoplasmic fractions from rat liver (LC; lane 1) and rat spleen (SC; lane 2) or mitochondrial fraction from rat spleen (SM; lane 3) with a 1:1000 dilution of anti-mito-C₁-THF synthase antibodies (anti-mito). Each lane contains 50 µg of total protein.

4.4 DISCUSSION

“The full-length 3.6-kb transcript is encoded in 28 exons. A shorter, 1.1-kb transcript is produced by an alternative splicing event, in which exon 7 is spliced to exon 8a instead of exon 8 (Figure 4.4). This transcript encodes a 275-amino acid protein in which the first 260 amino acids are identical to the full-length protein, followed by 15 amino acids not found in any other C₁-THF synthase. The first 11 amino acids of these 15 terminal amino acids are also found, with one mismatch, near the C terminus of isoform 2 of the human α 1A-adrenergic receptor, and the nucleotide sequence encoding these amino acids has high homology to an *Alu* repeat subfamily (Chang *et al.*, 1998). The first 91 nt of exon 8a share 87% identity with the right half of the consensus *Alu*-Sc subfamily (GenBankTM/EBI accession number U14571), suggesting that exon 8a was derived from an *Alu* element that inserted, in the antisense orientation, into the intron between exons 7 and 8. This insertion is not present in the mouse homolog because *Alu* elements are found only in primates (Sorek *et al.*, 2002). The exonization and alternative splicing of this *Alu* sequence in the human mitochondrial C₁-THF synthase gene are apparently due to accumulated mutations in the *Alu* element that produce a functional 3'-splice site (Lev-Maor *et al.*, 2003)”.

“One additional splicing variation was discovered. Some transcripts contained an extra codon at position +643, at the junction between exons 6

and 7 (Figure 4.5). This valine codon appears to arise from alternative usage of AG splice acceptor sites separated by 1 nt. This type of variation in splice site selection has been seen in several other mammalian genes, including human prothymosin- α (Manrow and Berger, 1993) and the rat transforming growth factor- β type I receptor (Agrotis *et al.*, 2000). The extra codon was observed in most of the 3'-RACE clones we sequenced and can be found in numerous human ESTs that represent the full-length transcript. There is no evidence to suggest that this alternative splice site selection is a regulated process. It may simply be due to "sloppiness" in the splicing mechanism when two AG splice acceptor sites fall so closely together".

“Based on the x-ray structure of the dehydrogenase/cyclohydrolase domain of the human cytoplasmic C₁-THF synthase (Schmidt *et al.*, 2000), the extra valine is predicted to reside on the exposed loop between α -helix D₂ and β -strand e. This loop is not part of the dehydrogenase/cyclohydrolase active site or the dimerization interface for this domain. It is thus possible that an extra valine at this position could be tolerated without affecting stability or activity of the protein. On the other hand, we do not know how the dehydrogenase/cyclohydrolase and synthetase domains interact, so it will be necessary to express the protein containing the extra valine to determine its effect”.

The short transcript was not expected to encode a functional protein *in vivo* since SI lacked exons 8 and 9 that encode the major portion of the Rossman fold of the NADP-binding site and a critical α -helix that forms one wall of the folate-binding site (Figure 4.8). Nonetheless, it was impossible to predict whether the 15 extra amino acids at the C-terminus of SI would facilitate it to fold into a stable protein with altered function or not. SI was expressed as a soluble fusion protein, MBP/SI, and the purified SI was stable in a buffer containing 20% glycerol. As expected, no NADP-dependent methylene-THF dehydrogenase activity was detected in the purified SI. Based on sequence alignment, the human cytoplasmic and mitochondrial isozymes share only 31% identity in the N-terminal dehydrogenase/cyclohydrolase domain when compared to 73% identity in the synthetase domain (Figure 2.4). We predicted that since SI represents just the N-terminus of the mitochondrial isozyme, polyclonal antibodies raised against it would be specific for the mitochondrial isozyme. However, the anti-mito-C₁-THF synthase antibodies show a slight signal with a band in rat liver cytosol (Figure 4.7; *Panel C, lane 2*), which may represent weak cross-reaction of these antibodies with the cytoplasmic isozyme. In comparison to the strong signal seen with these antibodies in rat spleen mitochondria (Figure 4.7; *Panel C, lane 3*), a small amount of cross-reaction does not interfere with its use to specifically detect mitochondrial C₁-THF synthase. At the same time, we were unable to detect the expression of any short isoform *in vivo* using human placental

mitochondria (see Chapter 5). Rat spleen mitochondria were not expected to give any signal since *Alu* elements are detected only in primates (Sorek *et al.*, 2002).

Once the 3'-end of the short transcript was determined, and the short isoform purified for polyclonal antibody synthesis, we focused on further characterization of the full-length protein. Chapter 5 describes the experiments conducted to determine the submitochondrial localization of human mitochondrial C₁-THF synthase.

```

      1      10      20      30      40      50      60      70      80      90      100     110
hmito  MGTALPLVLRLQLRPPQPPGPPRLALVPCRASSGGGGGGGGREGLLGQRRPQDGQARSSCSPGGRTPAAROSIVREVIQNSKEVLSIICPEKNPAREPDLATIQAG...DNLMOETINON
hcyto  .....MAPAEILNGKEISAOIARARLKNQVTCPEQVGGSTPLATIQVGNRDSNLYINYKLG

      120      130      140      150      160      170      180      190      200      210      220      230
hmito  LAEEMGLNITHICLPDSSSEETIDILKYNEDTRVHGALAI...SEN.LFSNKNVINALRPEKDVGCTDINLGAIVRGDAHECFVSPVAKAVIELLEKSGVNLDGKKILVVGAGHSLE
hcyto  RAEEICGKATHIKLERTTTSEVMKVITSINEDSTVHGFLVQLPLDSSENSINTEEVNNAIPEKDVGCTSINAGFURRGDINDCFIPECTPAGCLELIKEICGVPFAGRAHVVVGRSKIVG

      240      250      260      270
hmito  EALQCCFQRKGSMTMSIQKPKRQPSYTESRSVTRLECRRVI.....
hcyto  EPMHDDLWNNAVTTCSSKTAHDEEVNKGDDLVVATGQPEMVKGWIKPGAIVDCGINYVPDDKKPNGRKVVGQVAYDEAKERASFITPVFGG

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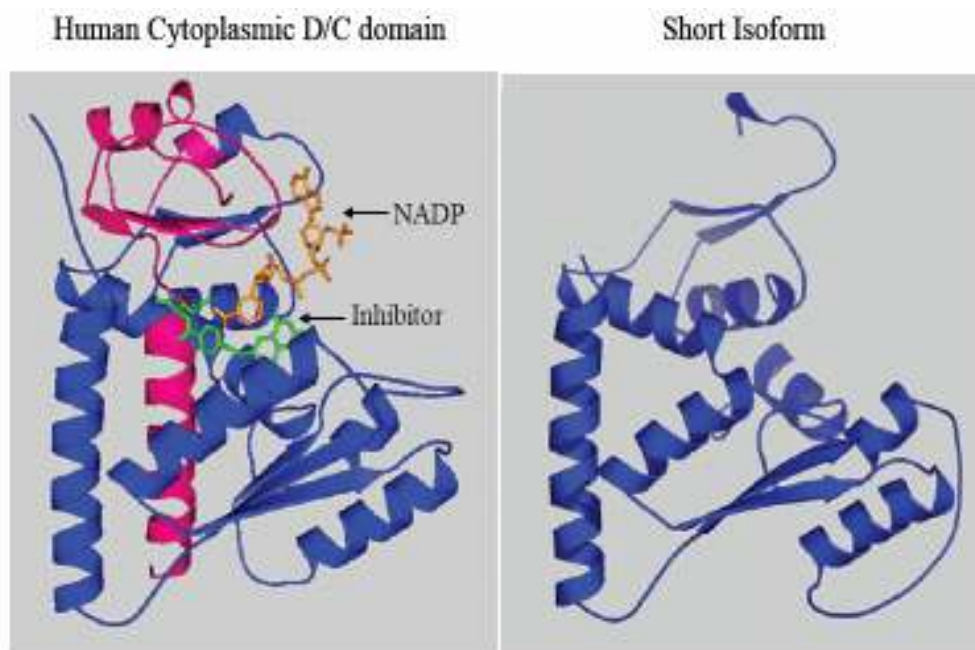


Figure 4.8 Alignment and Structure Comparison of the Short Isoform of Human Mitochondrial C₁-THF Synthase (*hmito*) and Human Cytoplasmic Dehydrogenase/Cyclohydrolase (D/C) Domain (*hcyto*). *Top*. Black boxes denote identity, and white boxes denote conservative substitutions. The C-terminal 15 amino acids of the short isoform are shown in rectangle. Asterisk indicate the first 'S' residue of purified short isoform. Alignment done as in Figure 2.4. *Bottom*. Crystal structure of D/C domain of human cytoplasmic C₁-THF synthase with bound NADP and inhibitor (*left panel*) (Schmidt *et al.*, 2000), and predicted protein model for the short isoform of human mitochondrial C₁-THF synthase (*right panel*). Model prediction done at <http://swissmodel.expasy.org/>

CHAPTER 5

Sublocalization of Human Mitochondrial C₁-THF Synthase

5.1 OVERVIEW

The full-length human mitochondrial C₁-THF synthase was shown to be localized to the mitochondria in CHO cells transfected with pcDNA3.1-humito (see Chapter 2). Earlier studies done in our lab had shown that adult rat liver mitochondria can metabolize carbon 3 of serine into formate by a folate-dependent pathway (Barlowe and Appling, 1988; Garcia-Martinez and Appling, 1993). To confirm that this one-carbon metabolism is occurring inside the mitochondria, we further explored the sublocalization of human mitochondrial C₁-THF synthase. To achieve this goal, submitochondrial fractionation experiments were conducted using the same mammalian expression system. Experiments were also performed to investigate membrane association and topology of the mitochondrial isozyme. Interestingly, human mitochondrial C₁-THF synthase appeared to be an inner mitochondrial membrane protein tightly associated with the matrix side of the inner membrane. The inner membrane localization of the mitochondrial isozyme was demonstrated in rat spleen mitochondria as well. *In vivo* expression levels of the protein was very high in rat spleen mitochondria while human placental mitochondria exhibited only a very low level of expression. Since the mammalian mitochondrial enzyme was shown to be monofunctional (Walkup and Appling, 2005) and given its

inner membrane localization, immunoprecipitation experiments were conducted in an attempt to isolate any potential interacting partners. Unfortunately, we were unable to detect any such interaction.

5.2 EXPERIMENTAL PROCEDURES

5.2.1 Mitochondrial Isolation and Submitochondrial fractionation from CHO/pcDNA3.1-humito cells

The GLYB line of CHO cells (Kao *et al.*, 1969; Kao and Puck, 1975) transfected with pcDNA3.1-humito (obtained from Dr. Barry Shane, University of California, Berkeley, CA) was used for all the experiments in this chapter, since this line gave more stable transfectants than the wild-type CHO cells used previously (see Chapter 2). The transfected cells were cultured in HyQ α -minimal Eagle's medium (HyClone) supplemented with 10% (v/v) fetal bovine serum, 0.25 mM glutamine, and 100 μ g/ml penicillin/streptomycin (Appendix IV). Mitochondria were isolated by disrupting the cells in a glass-teflon Thomas homogenizer using 20 up-and-down manual strokes (Appendix IV). The medium contained G418 Sulfate (0.8 mg/ml) for selection of transfectants.

The mitochondrial pellet was resuspended in homogenization solution (HMS; 0.25 M sucrose, 1 mM EGTA, 0.5% BSA and 10 mM HEPES-NaOH (pH 7.4)) without BSA and a Bradford assay was performed to determine protein concentration

(Appendix IV). Subfractionation of mitochondria was performed as essentially described for rat liver (Pedersen *et al.*, 1978). Mitoplasts were prepared by one of the two following methods. In the digitonin method (Pedersen *et al.*, 1978), mitochondria were stirred gently on ice for 15 min with digitonin (0.12 mg digitonin/mg mitochondria). The reaction was stopped by adding 3 volumes of HMS without BSA and centrifuged at 10,000 X g for 10 min. The supernatant (outer membrane/intermembrane space) was centrifuged at 100,000 X g for 1 h in a Beckman OptimaMax Ultracentrifuge using a TLA100.3 rotor. The final supernatant was the intermembrane space fraction and the pellet was resuspended in HMS without BSA to give the outer membrane fraction. The initial 10,000 X g pellet was washed by resuspending in twice the original volume of digitonin-treated mitochondria and centrifuged again at 10,000 X g to obtain the final pellet (Mitoplasts = matrix enclosed by inner membrane).

In the modified “swell-contract” method (Murthy and Pande, 1987; Pallotti and Lenaz, 2001), mitochondria were resuspended in 20 mM potassium phosphate (pH 7.2) and incubated on ice for 20 min. ATP and MgCl₂ were then added to a final concentration of 1 mM each and incubated for another 5 min on ice. The suspension was centrifuged at 10,000 X g for 10 min and the supernatant was saved in an ultracentrifuge tube. The pellet was resuspended in 2 ml of HMS without BSA and centrifuged at 10,000 X g for 10 min. The final pellet (mitoplasts) was saved on ice. The supernatant was combined with the first supernatant and centrifuged at 100,000

X g for 1 h. The supernatant was the intermembrane space fraction and the pellet was resuspended in HMS without BSA to yield the outer membrane fraction.

Mitoplasts from either method were resuspended in HMS without BSA, mixed with an equal volume of 0.025 M HEPES-KOH (pH 7.0) containing 1% Triton X-100 (0.5% final concentration), and incubated on ice for 30 min. Triton-treated mitoplasts were centrifuged at 100,000 X g for 1 h. The supernatant was the matrix and the pellet was resuspended in HMS without BSA to yield the inner membrane fraction. All the fractions from subfractionation experiments were stored at -70°C until use.

5.2.2 Immunoblotting of Submitochondrial Fractions

The protein concentration of the mitochondrial subfractions (outer membrane, intermembrane space, inner membrane and matrix) was determined using the Bradford assay. Forty µg of protein from mitochondria and its subfractions were separated by SDS-PAGE on a 7.5% or a 12% polyacrylamide gel for 50 min at 180 V. Immunoblotting was performed as described in Appendix IV. The primary antibody used for detection of the mitochondrial isozyme was mouse anti-V5 antibody (1:1000 dilution; Invitrogen) or rabbit anti-mito-C₁-THF synthase antibody (1:1000 dilution; see Chapter 4). Other antibodies were used as markers, including rabbit anti-Bcl-x_{S/L} (S-18) (1:1000 dilution; Santa Cruz Biotechnology) (Boise, 1993) for outer membrane, mouse anti-COXI (1:1000 dilution; Molecular Probes) for inner

membrane (Vijayvergiya *et al.*, 2005), and rabbit anti-Hsp60 (1:2000 dilution; Stressgen Biotechnologies) (Garesse and Vallejo, 2001) for inner membrane/matrix. Secondary antibody was either horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit antibody (1:2000 dilution; Zymed). Reacting bands were visualized by ECL Plus detection system (Amersham) as described in Appendix IV.

5.2.3 Alkaline Carbonate Extraction of CHO/pcDNA3.1-humito Mitochondria

Alkaline carbonate extraction of mitochondria was done essentially as described (Nair and McGuire, 2005). Briefly, mitochondria were obtained from CHO cells transfected with pcDNA3.1-humito (Appendix IV), and resuspended in HMS without BSA containing 1 mM PMSF and 1X protease inhibitor cocktail mixture (see appendix IV, protocol 10). Mitochondrial suspensions (100 μ l each) were treated with equal volumes of 1% Triton X-100 (0.5% final concentration) or 200 mM alkaline Na₂CO₃ (pH 11.5; 100 mM final concentration) or 266 mM NaCl (133 mM final concentration) and incubated on ice for 30 min. After centrifuging the samples at 150,000 X g for 30 min in a Beckman OptimaMax Ultracentrifuge using a TLA100.3 rotor, the supernatants were removed carefully and saved separately. The pellets were resuspended in 0.5% Triton X-100 or 100 mM Na₂CO₃ (pH 11.5) or 133 mM NaCl, respectively, to their initial volume (100 μ l) with protease inhibitors as above. The samples were incubated for another 30 min on ice and centrifugation was repeated. The supernatants from the two centrifugations were combined to give the

soluble fraction (S) and the pellets were resuspended in HMS without BSA to give the membrane fraction (P). All the fractions were stored at -70°C until use. The soluble and membrane fractions were subjected to SDS-PAGE and immunoblotting (Appendix IV) using antibodies anti-V5 (1:5000 dilution) and anti-mito-C₁-THF synthase (1:1000 dilution) for the mitochondrial isozyme, rabbit anti-MnSOD as a peripheral membrane protein marker (1:2000 dilution; Stressgen Biotechnologies) (Vijayvergiya *et al.*, 2005), and rabbit anti-porin as an integral membrane protein marker (1:2000 dilution; Calbiochem) (Ralphe *et al.*, 2004). Reacting bands were visualized by ECL Plus detection system.

5.2.4 Protease Treatment of CHO/pcDNA3.1-humito Mitochondria

Protease treatment of mitochondria was done essentially as described (Leary, 2004). Briefly, mitochondria were obtained from CHO cells transfected with pcDNA3.1-humito (Appendix IV), and resuspended in HMS without BSA. A Bradford assay was performed to determine the protein concentration of the resuspended mitochondria. Equal amounts of mitochondrial protein (~190 µg each) was transferred to different microfuge tubes and incubated with varying concentrations of digitonin (0.05 to 1 mg digitonin/mg protein) in the presence or absence of proteinase K (100 µg/ml) for 20 min on ice. Mitochondria incubated with 1% Triton X-100 in the presence of proteinase K were maintained as a positive control to test for the ability of proteinase K to digest the proteins of interest.

Digestion was stopped by adding PMSF (1 mM final concentration) and incubating on ice for another 20 min. After incubation, the mitochondrial suspensions were separately layered on top of a sucrose cushion (HMS without BSA containing 1 M sucrose) and centrifuged at 16,600 X g for 10 min. The supernatants were discarded and the pellets were resuspended in equal volumes of HMS without BSA for SDS-PAGE. In the Triton-treated controls, the pellet (P) and TCA-precipitated protein from the supernatant (S) were used for SDS-PAGE. Protein precipitation was done by adding 10% TCA to the supernatant and incubating at room temperature for 5 min. Precipitated proteins were pelleted by centrifugation at 16,600 X g for 10 min, and resuspended in HMS without BSA to give the soluble (S) fraction. All the fractions were stored at -70°C until use. Equal volumes of the different samples were subjected to SDS-PAGE and immunoblotting (Appendix IV) using anti-V5, anti-Hsp60, and anti Bcl-x_{S/L} antibodies at the same dilutions and secondary antibody as described in 5.2.2. Reacting bands were visualized by ECL Plus detection system.

5.2.5 Mitochondrial Isolation and Submitochondrial fractionation from Rat Spleen

Isolation and subfractionation of mitochondria from rat spleen was performed as described (Pedersen *et al.*, 1978) except for using a different buffer, H-medium (0.3 M sucrose, 1 mM EGTA, 5 mM MOPS, 5 mM KH₂PO₄, 0.1% BSA (pH 7.4)) (Rickwood *et al.*, 1987) (Appendix IV). The mitochondrial pellet was resuspended in

H-medium (0.3 M sucrose, 1 mM EGTA, 5 mM MOPS, 5 mM KH_2PO_4 , 0.1% BSA (pH 7.4)) without BSA and a Bradford assay was performed on the mitochondrial suspension. Subfractionation of mitochondria was done in the same way as for the cultured cells (see 5.2.1), except that in the “swell-contract” method, the outer membrane fraction was not separated from the intermembrane space fraction, and in the digitonin method, 0.3 mg digitonin/mg of mitochondrial protein was used. All the fractions from subfractionation experiments were stored at -70°C until use. The primary antibody used for detection of the mitochondrial isozyme in rat spleen was rabbit anti-mito- C_1 -THF synthase antibody (1:1000 dilution; see Chapter 4). Other antibodies were used as markers, including anti-Bcl- $\text{x}_{\text{S/L}}$ for outer membrane (1:2000 dilution), anti-COXI for inner membrane (1:1000 dilution), and anti-Hsp60 for inner membrane/matrix (1:2000 dilution). Reacting bands were visualized by ECL Plus detection system.

5.2.6 Isolation of Mitochondria from Human Placenta

Human placenta was kindly provided by St. David’s Medical Center (Austin, TX) and given to us by Dr. JoAnn Hunter Johnson (University of Texas, Austin). Isolation of mitochondria from human placenta was performed as described (Gray and Wong, 1992) (Appendix IV). A Bradford assay was performed to determine the protein concentration and the mitochondrial suspension was stored at -70°C until use.

5.2.7 Immunoprecipitation of Human Mitochondrial C₁-THF Synthase

CHO cells transfected with pcDNA3.1-humito, once confluent, were treated with 10 mM sodium butyrate (NaB) for 20 hrs to induce overexpression of the recombinant protein (Rizzardini *et al.*, 2005). Mitochondria were isolated, and immunoprecipitation was performed on mitochondrial lysate using Protein A sepharose beads and 1% NP-40 lysis buffer as described (Appendix IV). Mitochondrial lysate from untransfected CHO cells was used as a control. Immunoprecipitates were analyzed on a 12% SDS-polyacrylamide gel and silver stained as per Bio-Rad protocol.

5.3 RESULTS

5.3.1 CHO Cell Expression and Submitochondrial Localization

We previously showed that the protein expressed from pcDNA3.1-humito in CHO cells is localized to mitochondria (see Chapter 2). To determine the submitochondrial localization of this protein, mitochondria were isolated from transfected CHO cells and submitochondrial fractionation using the “swell-contract” or the digitonin method was performed (section 5.2.1). These submitochondrial fractions were subjected to SDS-PAGE and immunoblotting using antibodies against the V5 epitope and/or anti-mito-C₁-THF synthase antibodies to detect the human mitochondrial C₁-THF synthase and various mitochondrial marker proteins: Bcl-x_{S/L}

(outer membrane); COX I (inner membrane); and Hsp60 (inner membrane/matrix). The mitochondrial C₁-THF synthase was distributed equally between the inner membrane and matrix fractions (Figure 5.1A; *lanes 4 and 5*). Immunoblots of COXI (Figure 5.1A; *lane 4*) and Bcl-x_{S/L} (Figure 5.1B; *lane 2*) showed that the outer and inner mitochondrial membranes were not cross-contaminated.

Inner membrane and matrix fractions were separated by treating the mitoplasts with 0.5% Triton X-100. After Triton X-100 treatment, all of the COX I (57 kDa), an inner membrane marker was seen in the respective fraction itself (Figure 5.1A; *lane 4*) while most of Hsp60 (60 kDa), an inner membrane/matrix marker was released into the matrix (Figure 5.1A; *lane 5*). However, the mitochondrial isozyme (107 kDa), detected by the anti-V5 antibodies, was distributed equally between the inner membrane and matrix fractions (Figure 5.1A; *lanes 4 and 5*) suggesting that human mitochondrial C₁-THF synthase is at least partly associated with the mitochondrial inner membrane. Essentially the same result was obtained with anti-V5 and anti-mito-C₁-THF synthase antibodies on mitochondria fractionated using digitonin (Figure 5.1B; *lanes 4 and 5*).

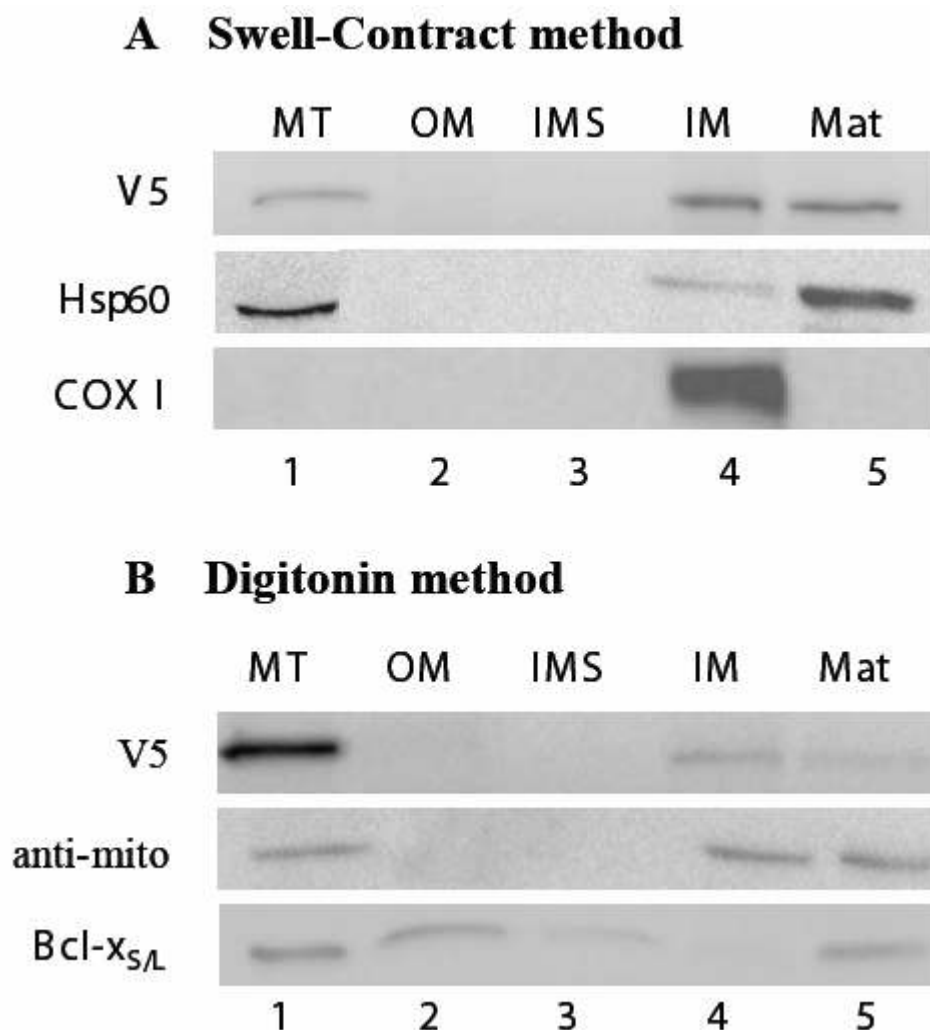


Figure 5.1 Submitochondrial Localization of Epitope-tagged Human Mitochondrial C₁-THF Synthase Expressed in CHO Cells. Mitochondria were subfractionated by “swell-contract” method (*Panel A*) or by digitonin method (*Panel B*). Shown are immunoblots of different fractions (whole mitochondria, MT; outer membrane, OM; intermembrane space, IMS; inner membrane, IM; and matrix, Mat) with anti-V5 or anti-mito-C₁-THF synthase or anti-Hsp60 (inner membrane/matrix marker) or anti-COX I (inner membrane marker) or anti-Bcl-x_{S/L} (outer membrane marker) antibodies. Antibody dilutions are given in 5.2.1. Each lane contains forty µg of total protein.

5.3.2 Alkaline Carbonate Extraction of CHO/pcDNA3.1-humito Mitochondria

To determine the nature of the membrane association, mitochondria were subjected to alkaline (pH 11.5) sodium carbonate treatment (section 5.2.3). Alkaline sodium carbonate is known to release peripheral membrane proteins into the soluble fraction while integral membrane proteins are retained in the insoluble membrane fraction (Fujiki *et al.*, 1982). The integral outer membrane marker protein, porin (31 kDa), was observed in the pellet (membrane fraction; *lane 5*) (Figure 5.2) whereas almost all of the peripheral membrane marker protein, MnSOD (25 kDa), was observed in the supernatant (soluble fraction; *lane 6*). In contrast to MnSOD, the mitochondrial isozyme (107 kDa) was distributed equally between the membrane and soluble fractions (*lanes 5 and 6*) detected by both anti-V5 and anti-mito-C₁-THF synthase antibodies. Mitochondria treated with NaCl of the same ionic strength as Na₂CO₃ failed to release the mitochondrial isozyme from the membrane fraction (*lanes 3 and 4*). Triton X-100 treatment also released only about half of the mitochondrial C₁-THF synthase from the membrane (*lanes 1 and 2*). MnSOD, although a matrix marker, has been reported to behave partly as an insoluble membrane protein (Nair and McGuire, 2005), even in the presence of detergent (Vijayvergiya *et al.*, 2005), explaining its presence in the membrane fraction even after treatment with Triton X-100 (*lane 1*). These data suggest that human mitochondrial C₁-THF synthase is a tightly associated peripheral membrane protein of the mitochondrial inner membrane.

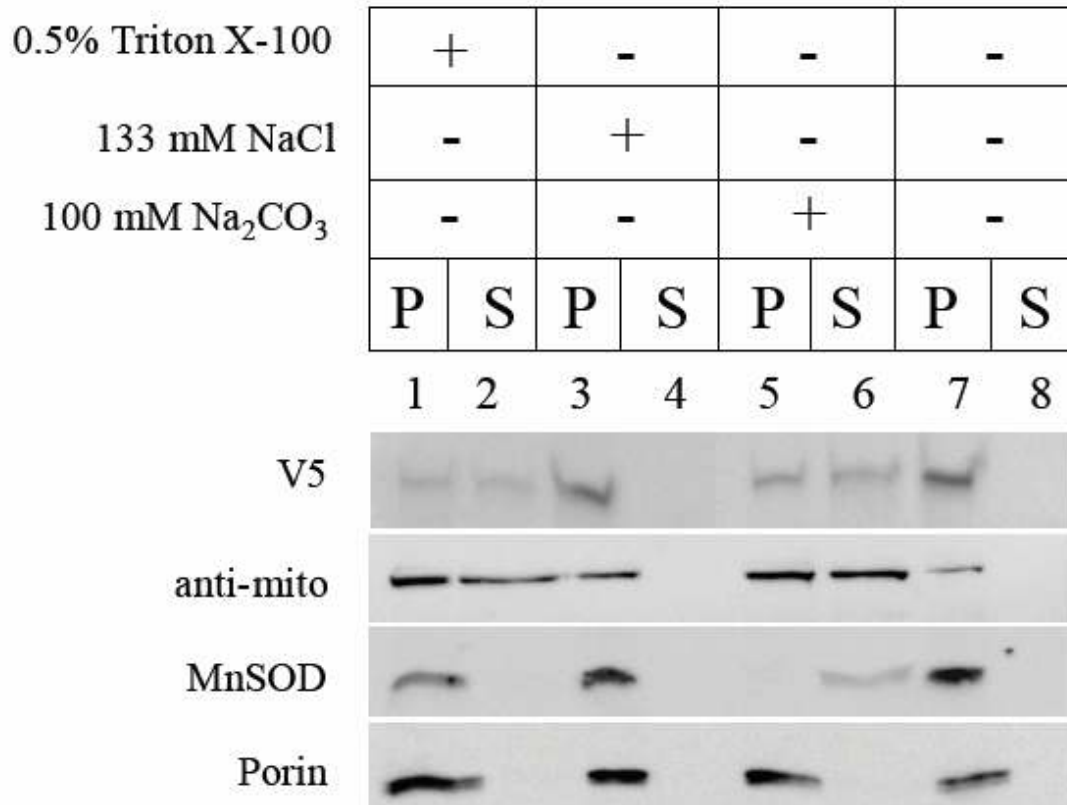


Figure 5.2 Alkaline Carbonate Extraction of CHO/pcDNA3.1-humito Mitochondria. Mitochondria were treated with Na₂CO₃ (pH 11.5) or NaCl or Triton X-100. Shown are immunoblots of the insoluble integral membrane proteins (pellet; *P*) and the soluble peripheral membrane proteins (supernatant; *S*) with anti-V5 or anti-mito C₁-THF synthase or anti-MnSOD (matrix marker) or anti-porin (outer membrane marker) antibodies. Antibody dilutions are given in 5.2.3. Each lane contains the same volume of sample.

5.3.3 Membrane Topology of Human Mitochondrial C₁-THF Synthase

Submitochondrial fractionation and alkaline carbonate extraction experiments suggested that human mitochondrial C₁-THF synthase is tightly associated with the inner membrane. To determine the topology of the mitochondrial isozyme within the mitochondrial inner membrane, mitochondria were treated with increasing amounts of digitonin in the presence or absence of 100 µg/ml proteinase K (section 5.2.4). Immunoblotting was performed using anti-V5, anti-Hsp60 (matrix marker), and anti-Bcl-x_{S/L} (outer membrane marker) antibodies. The digestion pattern of mitochondrial C₁-THF synthase was different from the outer membrane marker but closely resembled the matrix marker (Figure 5.3; *A and B*). Mitochondrial C₁-THF synthase behaved essentially the same in the presence or absence of proteinase K, showing that digitonin does not make it susceptible to proteolysis. However, at the highest digitonin concentration, most of the mitochondrial isozyme was solubilized, but not the matrix marker Hsp60. On the other hand, Bcl-x_{S/L} antibodies gave two bands (35 kDa and 32 kDa) with different digestion patterns, the longer form being resistant to proteinase K digestion even after Triton treatment. Although the expected molecular weight of Bcl-x_L and Bcl-x_S are 30 kDa and 23 kDa respectively, anti-Bcl-x_{S/L} antibodies detected 35 kDa and 32 kDa proteins in CHO cell mitochondria. This variation in molecular weights has been observed by others at least for the longer form of Bcl-x (Alonso *et al.*, 1997). The shorter form was digested by proteinase K at a low concentration of digitonin, as expected for an outer membrane marker.

These data suggest that human mitochondrial C₁-THF synthase is associated with the matrix side of the inner membrane.

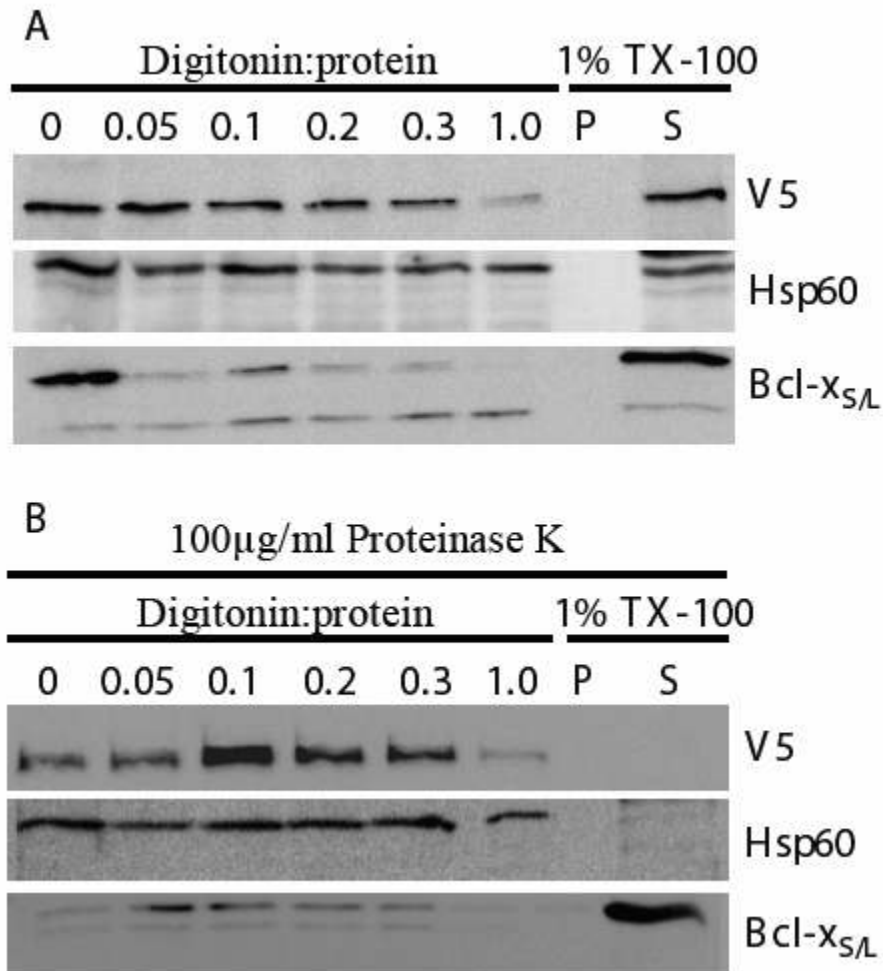


Figure 5.3 Membrane Topology of Human Mitochondrial C₁-THF Synthase Expressed in CHO Cells. Equal amounts of mitochondria (190 μ g) were incubated with varying concentrations of digitonin (0.05-1 mg digitonin/mg protein) in the absence (*panel A*) or presence (*panel B*) of proteinase K (100 μ g/ml). Shown are immunoblots of 20 μ l each of mitochondria with anti-V5 or anti-Hsp60 (matrix marker) or anti-Bcl-x_{S/L} (outer membrane marker) antibodies. Mitochondria incubated with 1% Triton X-100 in the presence or absence of proteinase K were used as positive and negative controls, respectively. In these controls, the pellet (*P*) and TCA-precipitated proteins from the supernatant (*S*) were used. Antibody dilutions are given in 5.2.4.

5.3.4 Submitochondrial Localization of Rat Spleen Mitochondrial C₁-THF Synthase

We next asked whether the inner mitochondrial membrane localization of human mitochondrial C₁-THF synthase observed in CHO cells holds true in mammalian tissues. Rat spleen mitochondria were subjected to fractionation and proteins were immunoblotted with anti-mito-C₁-THF synthase or the different marker antibodies (section 5.2.5). In both the “swell-contract” (Figure 5.4A) and digitonin methods (Figure 5.4B), the rat mitochondrial isozyme was seen partitioned between the inner membrane and matrix fractions (Figure 5.4A; *lanes 3 and 4*; Figure 5.4B; *lanes 4 and 5*). Most of the inner membrane/matrix marker protein, Hsp60, was released into the matrix (Figure 5.4A; *lane 4*), and the inner membrane marker, COX I, was abundant in the inner membrane fraction (Figure 5.4A; *lane 3*). Immunoblotting using the outer membrane marker (anti-Bcl-x_{S/L}) antibody showed that the inner membrane fraction was not contaminated with outer membrane (Figure 5.4B; *lanes 2 and 4*). A less intense signal for Bcl-x_{S/L} was seen in the intermembrane space. Thus, like in the cultured cells, the mitochondrial isozyme appears to be associated with the inner membrane in adult rat spleen mitochondria as well.

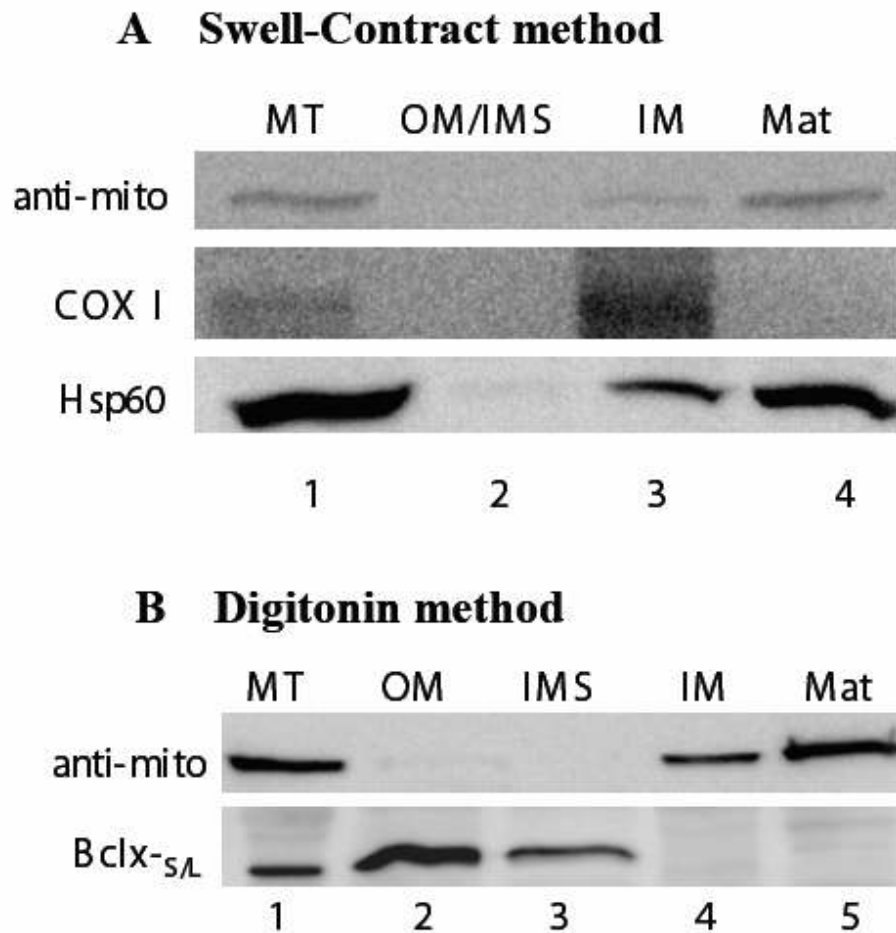


Figure 5.4 Submitochondrial Localization of Endogenous Rat Spleen Mitochondrial C₁-THF Synthase. Mitochondria were subfractionated by the “swell-contract” method (*Panel A*) or by the digitonin method (*Panel B*). Shown are immunoblots of different fractions (whole mitochondria, MT; outer membrane/intermembrane space, OM/IMS, or outer membrane, OM; intermembrane space, IMS; inner membrane, IM; and matrix, Mat) with anti-mito-C₁-THF synthase or anti-COX I (inner membrane marker) or anti-Hsp60 (inner membrane/matrix marker) or anti-Bcl-x_{S/L} (outer membrane marker) antibodies. Antibody dilutions are given in 5.2.5. Each lane contains thirty μ g of total protein.

5.3.5 Expression Level of Mitochondrial C₁-THF Synthase in Tissues and CHO Cells

Mitochondria from rat spleen, human placenta and CHO/pcDNA3.1-humito cells (100 µg) were fractionated on a 7.5% SDS-polyacrylamide gel in duplicates. One half of the gel was Coomassie-Blue stained (Figure 5.5; *left panel*) and the other half of the gel was subjected to immunoblotting with anti-mito C₁-THF synthase antibodies (Figure 5.5; *right panel*). Rat spleen and CHO/pcDNA3.1-humito cells (*lanes 1 and 3*) gave an intense signal of ~100 and ~107 kDa, respectively. Mitochondria from human placenta also gave a good signal (*lane 2*) although less intensive than the other two, thereby providing evidence for the *in vivo* expression of human mitochondrial C₁-THF synthase. No signal of the size of the short isoform of mitochondrial C₁-THF synthase was detected in placental mitochondria.

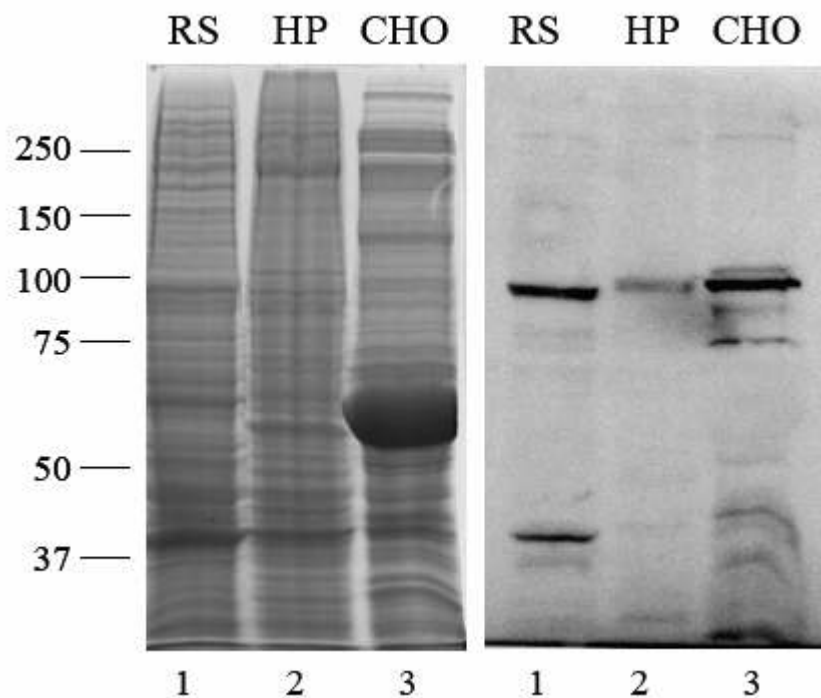


Figure 5.5 Expression Level of Mitochondrial C₁-THF Synthase. Coomassie-Blue stained 7.5% SDS-PAGE (left panel) and Immunoblot (right panel) of mitochondrial fractions from rat spleen (RS; *lane 1*), human placenta (HP; *lane 2*), and CHO/pcDNA3.1-humito (CHO; *lane 3*). A 1:1000 dilution of anti-mito C₁-THF synthase antibodies was used. Each lane contains 100 µg of total protein. Locations and size (kDa) of molecular weight markers are shown on the left.

5.3.6 Immunoprecipitation of Human Mitochondrial C₁-THF Synthase

Enzyme assays on purified recombinant human mitochondrial C₁-THF synthase showed that the enzyme is monofunctional, possessing only the 10-formyl-

THF synthetase activity (Walkup and Appling, 2005). Yet mitochondria possess the ability to oxidize one-carbon units from the level of 5,10-CH₂-THF to 10-formyl-THF (Garcia-Martinez and Appling, 1993). This suggests the existence of another protein that provides the missing 5,10-methylene-THF-dehydrogenase and 5,10-methenyl-THF-cyclohydrolase activities. Immunoprecipitation experiments were performed to see whether the mitochondrial isozyme interacts with any protein.

CHO cells transfected with pcDNA3.1-humito do not overexpress the human mitochondrial C₁-THF synthase. To ensure abundant levels of the recombinant protein for immunoprecipitation, cells were treated with sodium butyrate (NaB). NaB is known to activate the CMV promoter thereby inducing overexpression of proteins under its control (Rizzardini *et al.*, 2005). An immunoblot of uninduced (*lane 1*) and induced (*lane 2*) cells with anti-V5 antibodies (1:5000 dilution) showed that NaB indeed caused overexpression of the mitochondrial isozyme (Figure 5.6, *Panel A*). The human mitochondrial C₁-THF synthase was immunoprecipitated from mitochondrial extract using Protein A sepharose beads and anti-V5 monoclonal antibodies. A silver-stained gel of the immunoprecipitate from transfected CHO cells gave 3 protein bands: 107 kDa (mitochondrial C₁-THF synthase), 50 kDa (IgG heavy chain), and an unknown protein at 200 kDa. However, immunoprecipitate from untransfected CHO cells also gave the 200 kDa band along with the IgG heavy chain band. No other significant bands were seen in immunoprecipitate from transfected CHO cells.

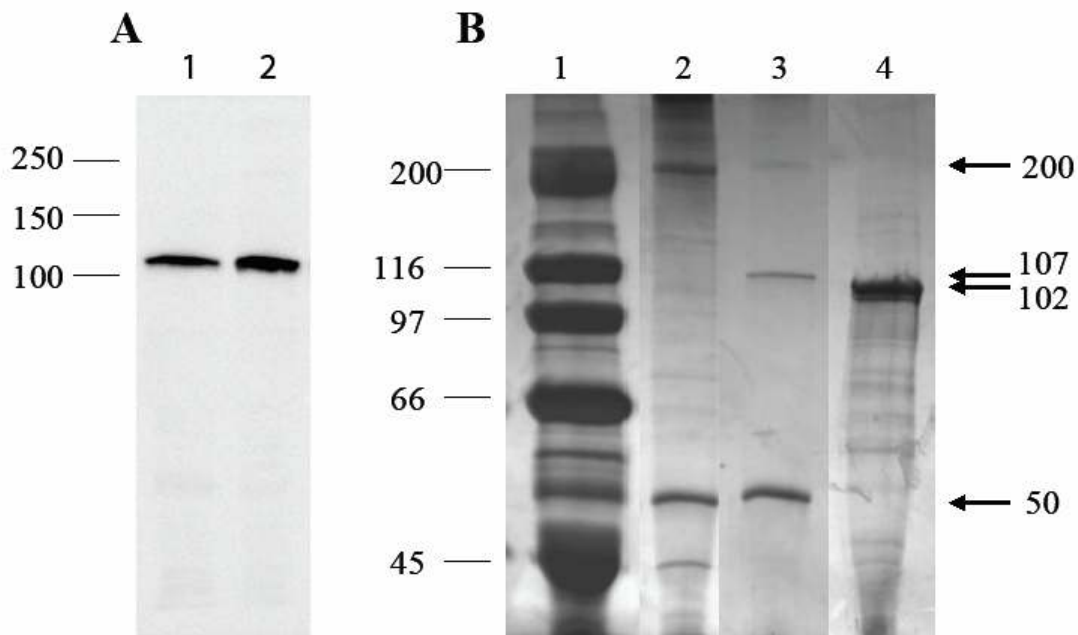


Figure 5.6 Immunoprecipitation of Human Mitochondrial C₁-THF Synthase. Panel A. Immunoblot of mitochondria from uninduced (*lane 1*) and NaB induced (*lane 2*) CHO cells transfected with pcDNA3.1-humito with anti-V5 antibodies. Each lane contains 50 μ g of mitochondrial total protein. Panel B. Silver-stained SDS-polyacrylamide gel of immunoprecipitates (30 μ l each) from CHO cells transfected with pcDNA3.1-humito (*lane 3*) or an untransfected control (*lane 2*). Lane 1 contains molecular weight markers. Lane 4 contains 1 μ g of purified recombinant protein (Walkup and Appling, 2005). Molecular weight markers are given in kDa.

5.4 DISCUSSION

Subfractionation of mitochondria from CHO cells transfected with pcDNA3.1-humito showed that human mitochondrial C₁-THF synthase is a mitochondrial inner membrane protein (Figure 5.1). Separation of outer membranes from intact mitoplasts was achieved using either the “swell-contract” or the digitonin method. Immunoblots of COX I and Bcl-x_{SL} showed that the subfractionation was successful in achieving enrichment of the respective compartments (Figure 5.1; *A and B*). Most of Hsp60, a matrix marker, was released into the matrix (Figure 5.1; *A*). A weak signal of Hsp60 seen in the inner membrane can be explained by the observation that Hsp60 is one of several mitochondrial DNA-binding proteins that associates with the inner membrane (Garesse and Vallejo, 2001). Similar results obtained with rat spleen mitochondrial subfractionation indicate that mitochondrial C₁-THF synthase is associated with the inner membrane in adult mammalian tissues as well (Figure 5.4; *A and B*). Other folate-dependent mitochondrial enzymes including the glycine cleavage system and serine hydroxymethyltransferase have been reported to be associated with the inner membrane in rat liver mitochondria (Motokawa and Kikuchi, 1971). Since these two enzymes catalyze the formation of 5,10-methylene-THF, the finding that the mitochondrial C₁-THF synthase is inner membrane associated seems quite significant. The yeast mitochondrial C₁-THF synthase has generally been assumed to be a soluble matrix enzyme. However, it was purified from yeast cell extract prepared by mechanical disruption followed by a low

speed centrifugation (any membrane fraction will not sediment under these conditions) to remove any unbroken cells (Shannon and Rabinowitz, 1986). Since it is evident that the human mitochondrial enzyme is an inner membrane protein, it would be interesting to analyze whether the yeast mitochondrial enzyme shows any inner membrane association.

Alkaline carbonate (pH 11.5) extraction of mitochondria has been shown to solubilize peripheral membrane proteins while retaining integral membrane proteins in the insoluble fraction (Fujiki *et al.*, 1982). In contrast to the membrane marker, porin, and matrix marker, MnSOD, which were seen in the insoluble and soluble fractions, respectively, mitochondrial C₁-THF synthase was equally distributed between the two fractions (Figure 5.2). This result was similar to that observed for mitochondrial folylpolyglutamate synthetase (FPGS) (Nair and McGuire, 2005). These authors proposed that the inner membrane localization of FPGS would increase the efficiency of polyglutamylation of folates by promoting substrate channeling between the mitochondrial folate transport protein and FPGS. It was evident that the extraction occurs only at high pH since NaCl of the same ionic strength failed to solubilize the mitochondrial isozyme. The pH specificity of the alkaline carbonate extraction of membrane proteins has been reported before (Fujiki *et al.*, 1982). The human mitochondrial isozyme was not completely susceptible to alkaline carbonate extraction, suggesting a tight association with the inner membrane. However, computer analysis for transmembrane proteins

(http://www.ch.embnet.org/software/TMPRED_form.html) failed to predict the presence of any transmembrane regions in mitochondrial C₁-THF synthase. On the other hand, the human mitochondrial isozyme has a 62 amino acid N-terminal extension, of which only the first 31 amino acids represent the mitochondrial targeting sequence (Sugiura *et al.*, 2004). The mature form of the enzyme retains the remaining 31 glycine/arginine rich amino acids that are not seen in either the cytoplasmic or the yeast mitochondrial C₁-THF synthases. The function of this N-terminal extension that is also seen in the mouse and rat homologs is not known so far. It is not responsible for the loss of the dehydrogenase and/or cyclohydrolase activities of the mitochondrial isozyme, as a recombinant enzyme lacking the N-terminal extension failed to regain either of these activities (unpublished data from our lab). Other possibilities for the function of the N-terminal extension include potential involvement in either anchoring the enzyme to the inner membrane or in protein-protein interactions. The former possibility seems likely due to the amino acid composition of the N-terminal extension, and especially since we have shown that the mitochondrial C₁-THF synthase is tightly associated with inner mitochondrial membrane. The N-terminal extension has a stretch of nine glycines. In plant chloroplasts, it has been shown that removal of a polyglycine stretch targets a transport channel protein to the stroma instead of the outer envelope membrane (Inoue and Keegstra, 2003). Submitochondrial localization of mitochondrial C₁-THF synthase lacking the 31 amino acid N-terminal extension will tell us whether the

polyglycine stretch plays a role in localizing the protein in the inner membrane. Any involvement of the N-terminal extension in facilitating protein-protein interactions needs to be investigated as well.

Proteinase K treatment of mitochondria shows that the human mitochondrial C₁-THF synthase faces the matrix side of the inner membrane (Figure 5.3). The matrix orientation of the mitochondrial isozyme may suggest potential protein-protein interactions that may include a hypothetical 5,10-methylene-THF dehydrogenase. If so, the inner membrane association and matrix orientation may be beneficial to enable efficient substrate channeling between the potential dehydrogenase and C₁-THF synthase, as suggested for FPGS (Nair and McGuire, 2005). Folate-dependent enzymes are known to exist in multienzyme complexes in yeast as well as in mammals for facilitating various metabolic processes (Appling, 1991). The possibility of mitochondrial C₁-THF synthase forming a multienzyme complex with other folate-dependent enzymes in the inner mitochondrial membrane (for example, serine hydroxymethyltransferase and/or glycine cleavage system) cannot be ruled out.

It has been proposed that the synthetase activity of mitochondrial C₁-THF synthase together with the dehydrogenase/cyclohydrolase activities of the bifunctional enzyme may complete one-carbon metabolism in embryonic and transformed cells (Christensen *et al.*, 2005). A Japanese group reported that the mitochondrial C₁-THF synthase gene was found to be overexpressed in human cancer cells (Sugiura *et al.*, 2004). In light of this evidence, they proposed that upregulation

of the mitochondrial isozyme may support cell proliferation. However, none of these can explain the metabolism of carbon 3 of serine into formate that was observed in adult rat liver mitochondria (Garcia-Martinez and Appling, 1993). Recently, studies by other members of the lab revealed that the recombinant human mitochondrial C₁-THF synthase is monofunctional, possessing only the 10-formyl-THF synthetase activity (Walkup and Appling, 2005). Since mitochondrial C₁-THF synthase is associated with the matrix side of the inner membrane (current study) and it is monofunctional (Walkup and Appling, 2005), we hypothesize the existence of an interacting protein that may provide the missing activities. Initial attempts to detect any protein-protein interactions of human mitochondrial C₁-THF synthase were not successful (Figure 5.6B). This could be because any interaction, if present, may not be stable under the immunoprecipitation conditions chosen. The use of lower concentrations of the detergent or different detergents or Protein G Sepharose beads or varying the salt concentration in the lysis buffer could be tested in future experiments. Alternative methods such as Blue-Native PAGE could also be applied to detect potential interacting partners that could explain the conversion of 5,10-methylene-THF to 10-formyl-THF in adult mammalian mitochondria.

Northern blot analysis of mitochondrial C₁-THF synthase transcripts in adult human tissues showed high expression of the gene in spleen and placenta (see Chapter 3). Likewise, rat spleen mitochondria showed a high level of expression at the protein level compared to CHO cells. Also, the mitochondrial isozyme is

detectable on immunoblot of human placental mitochondria (Figure 5.5), indicating that mitochondrial C₁-THF synthase is expressed at the protein level *in vivo* in humans as well. Immunoblots of at least two different human cancer cell lines using anti-mito-C₁-THF synthase antibodies have also shown the expression of mitochondrial isozyme (Schuyler Pike, personal communication). We were unable to detect any signal corresponding to the size of the short isoform in human placental mitochondria. Thus, it appeared that the short transcript was either not translated *in vivo* or if translated, it might not have folded into a stable structure and was rapidly degraded. When immunoblots were performed on total cell extract from both tissues, rat spleen gave a faint signal while human placenta failed to give any signal of the mitochondrial isozyme (data not shown).

This research has given us valuable information on mammalian mitochondrial C₁-THF synthase. The existence of a mammalian homolog of the yeast mitochondrial trifunctional enzyme was controversial several years ago. We have shown that the human genome encodes a C₁-THF synthase that is localized in the mitochondrial inner membrane in a mammalian cell line, and the same is true in adult mammalian tissues. Although the gene was found to be expressed in all human tissues examined, human placenta (the only human tissue examined for protein) failed to show high expression of the transcript as opposed to rat spleen mitochondria. The existence of mammalian mitochondrial C₁-THF synthase with a potential interacting partner/s or maybe as a multienzyme complex will await further biochemical characterization.

CHAPTER 6

CONCLUDING REMARKS

6.1 Dissertation Summary

At the beginning of this project, the existence, structure, and function of the folate-interconverting activities of C₁-THF synthase in mammalian mitochondria was controversial. Previous research performed in adult rat liver mitochondria had suggested the involvement of a mitochondrial C₁-THF synthase in supporting cytoplasmic one-carbon metabolism (Barlowe and Appling, 1988; Garcia-Martinez and Appling, 1993), but the enzyme was never isolated. In the course of this project, a full-length cDNA clone (pcDNA3.1-humito) of human mitochondrial C₁-THF synthase was constructed, and cloned into the mammalian expression vector pcDNA3.1D/V5-His-TOPO. The expression of the full-length cDNA clone in CHO cells showed that the protein encoded was targeted exclusively to the mitochondria. Through Northern analysis, the gene was found to be expressed in most human tissues examined, and was found to be distinct from the cytoplasmic isozyme. The transcriptional start site was identified as well. In addition to the full-length transcript, a shorter transcript was observed that was shown to arise as a result of alternative splicing. A cDNA clone of the short transcript was constructed, expressed in *E. coli*, and purified. This protein, although catalytically inactive, was used to make polyclonal antibodies specific for the mitochondrial C₁-THF synthase isozyme.

To confirm that the one-carbon metabolism observed in adult rat liver is occurring inside the mitochondria, mitochondrial subfractionation experiments were conducted to better define the localization of mitochondrial C₁-THF synthase. Interestingly, the mitochondrial isozyme was found to be tightly associated with the matrix side of the inner mitochondrial membrane. It has been reported that the human mitochondrial folylpolyglutamate synthetase (FPGS) is associated with the inner membrane as well (Nair and McGuire, 2005). They have suggested that the membrane association may increase the efficiency of polyglutamylation of folates by allowing substrate channeling between the mitochondrial folate-transport protein and FPGS. We hypothesize that the human mitochondrial C₁-THF synthase may have an interacting partner that may provide the missing activities (5,10-methylene-THF dehydrogenase and 5,10-methenyl-THF cyclohydrolase) and if so, the inner membrane localization may enable efficient substrate channeling between the two enzymes. However, initial efforts to co-immunoprecipitate proteins that may interact with human mitochondrial C₁-THF synthase were not successful. A figure summarizing these results and depicting our current model is shown (Figure 6.1).

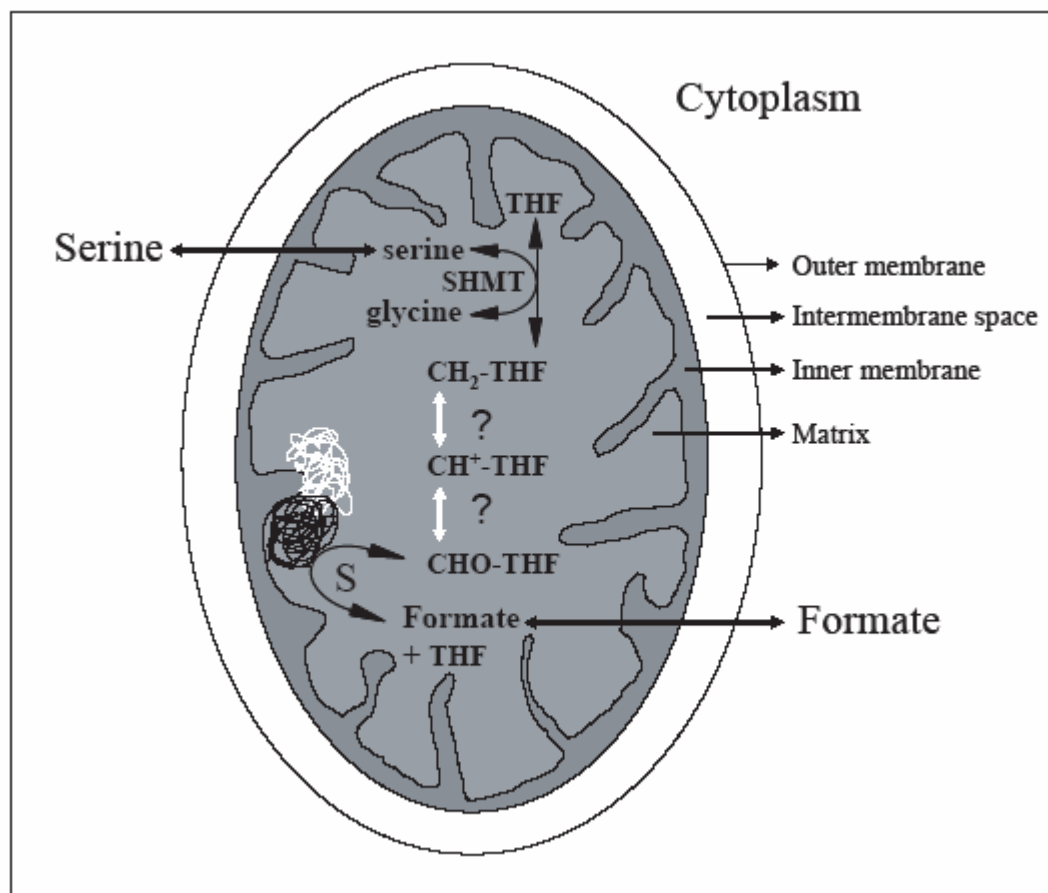


Figure 6.1 Proposed Model for THF-mediated One-Carbon Metabolism in Adult Mammalian Mitochondria. THF accepts a one-carbon unit from serine to form $\text{CH}_2\text{-THF}$. The synthetase activity of mitochondrial $\text{C}_1\text{-THF}$ synthase (shown in black) together with the dehydrogenase/cyclohydrolase activities of an interacting enzyme (shown in white) converts $\text{CH}_2\text{-THF}$ to formate, releasing THF. Formate exits the mitochondria to serve as one-carbon donor in cytoplasmic processes. The abbreviations used are: THF, tetrahydrofolate; SHMT, serine hydroxymethyltransferase; $\text{CH}_2\text{-THF}$, 5,10-methylene-THF; $\text{CH}^+\text{-THF}$, 5,10-methenyl-THF; CHO-THF , 10-formyl-THF.

6.2 Conclusions and Future Work

Adult rat liver mitochondria have been shown to oxidize the 3rd carbon of serine to formate and CO₂ (Barlowe and Appling, 1988; Garcia-Martinez and Appling, 1993). This pathway produces 5,10-methylene-THF, which must then be oxidized to 5,10-methenyl-THF followed by its conversion to 10-formyl-THF (see Figure 1.4). 10-formyl-THF can be either oxidized to CO₂ and THF or converted to formate and THF. In *Saccharomyces cerevisiae*, the oxidation of 5,10-methylene-THF is catalyzed by 5,10-methylene-THF dehydrogenase activity of the mitochondrial trifunctional C₁-THF synthase (Shannon and Rabinowitz, 1986; Shannon and Rabinowitz, 1988). However, the nature of this activity in mammalian mitochondria remains unclear. During the course of this work, research by other members of our laboratory revealed that the gene product of the human mitochondrial C₁-THF synthase was monofunctional, possessing only the 10-formyl-THF synthetase activity (Walkup and Appling, 2005). This is interesting since all C₁-THF synthases characterized so far, from yeast to mammals, are trifunctional. Furthermore, MacKenzie and coworkers reported in embryonic fibroblasts that cytoplasmic C₁-THF synthase knockouts exhibit only the 10-formyl-THF synthetase activity of the mitochondrial C₁-THF synthase (Christensen *et al*, 2005). Based on sequence alignment of the mitochondrial isozyme with other trifunctional C₁-THF synthases, the loss of dehydrogenase (D) and cyclohydrolase (C) activities have been attributed to amino acid substitutions in the N-terminal D/C domain of human

mitochondrial C₁-THF synthase in key residues required for cofactor binding or directly involved in the cyclohydrolase activity (Christensen *et al.*, 2005). Expression of the mitochondrial isozyme from a construct in which the N-terminal amino acid substitutions have been reversed will determine their effect on the lost dehydrogenase/cyclohydrolase activities.

In addition to serine mentioned above, adult rat liver mitochondria can synthesize formate and CO₂ from N-methyl carbon of sarcosine (Mitoma and Greenberg, 1952; Frisell and Sorell, 1967; Lewis *et al.*, 1978; Barlowe and Appling, 1988) as well as the second carbon of glycine (Anne Tibbetts, personal communication). All three one-carbon donors, glycine, serine, and sarcosine, produce the common intermediate 5,10-methylene-THF, catalyzed by the glycine cleavage system, serine hydroxymethyltransferase, and sarcosine dehydrogenase, respectively. Among these, the glycine cleavage system and sarcosine dehydrogenase are strictly mitochondrial. Oxidation of 5,10-methylene-THF to 10-formyl-THF releases a pair of electrons, which would eventually enter the electron transport chain (ETC). Rotenone, an inhibitor of Complex I of ETC, was shown to inhibit the production of CO₂ (an NADP-dependent reaction), but not of formate, from serine in actively respiring mitochondria (Garcia-Martinez and Appling, 1993). If conversion of 5,10-methylene to 10-formyl-THF is an NAD- or NADP-dependent reaction, rotenone would be expected to inhibit formate production as well. The inability of rotenone to inhibit formate production may suggest that the dehydrogenase activity

that we are looking for is either not NAD- or NADP-dependent or it is not tightly coupled to ETC. To date, the human genome is known to encode three folate-interconverting isozymes: the cytoplasmic trifunctional C₁-THF synthase on chromosome 14, mitochondrial monofunctional C₁-THF synthase on chromosome 6, and mitochondrial bifunctional dehydrogenase/cyclohydrolase on chromosome 2. Also, there is a C₁-THF synthase pseudogene present on chromosome X. The bifunctional enzyme, along with the synthetase activity of mitochondrial C₁-THF synthase, has been proposed to support folate-dependent one-carbon metabolism in embryonic and transformed mammalian cells (Christensen *et al.*, 2005). However, folate-dependent one-carbon metabolism in adult mammalian mitochondria is not clear. Our results suggest that another enzyme capable of converting mitochondrial 5,10-methylene-THF to 10-formyl-THF may be present in mammalian mitochondria, although the human genome contains no other open reading frame homologous to the known 5,10-methylene-THF dehydrogenases. Currently, efforts are underway to identify this activity using serine, sarcosine, and glycine as one-carbon donors in combination with inhibitors of different complexes of ETC. Subfractionation experiments have proved that the mammalian mitochondrial C₁-THF synthase is tightly associated with the inner mitochondrial membrane. Given the fact that the mitochondrial isozyme has an unusual N-terminal extension, its involvement in membrane association/protein interaction needs to be investigated. Although initial

efforts to detect protein interactions with mitochondrial C₁-THF synthase failed, more sensitive detection methods may reveal these types of interactions.

In summary, this dissertation explains in depth the identification of the gene encoding the human mitochondrial C₁-THF synthase, and its cloning and characterization. The protein encoded by this gene was shown to be localized in the mitochondria, specifically in the inner mitochondrial membrane in a mammalian cell line. This exciting research has brought into light the existence of a mitochondrial C₁-THF synthase, the existence of which had been controversial. Isolation of an interacting protein possessing 5,10-methylene-THF dehydrogenase activity would complete the proposed model for one-carbon metabolism in adult mammalian mitochondria.

APPENDIX I

Abbreviations

AICAR	aminoimidazole carboxamide ribonucleotide
Amp	ampicillin
ATP	Adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
C ₁	one-carbon
Cam	chloramphenicol
cDNA	complementary DNA
CHO	Chinese hamster ovary
CH ⁺ -THF	5,10-methenyltetrahydrofolate
CH ₂ -THF	5,10-methylenetetrahydrofolate
CHO-THF	10-formyltetrahydrofolate
CIP	calf intestinal phosphatase
CMV	cytomegalovirus
cpm	counts per minute
D/C	dehydrogenase/cyclohydrolase
ddH ₂ O	distilled, deionized water
DHFR	dihydrofolate reductase
DMSO	dimethyl sulfoxide
dNTP	deoxyribonucleotide triphosphate
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
ETC	electron transport chain
FBS	fetal bovine serum

GAR	glycinamide ribonucleotide
GC	guanine cytosine
GCS	glycine cleavage system
HEPES	N-(2-hydroxyethyl)-piperazine-N'-(2-ethanesulfonic acid)
His	histidine
HMS	homogenization solution
IPTG	isopropyl- β -D-thiogalactopyranoside
kbp	kilo base pair
kDa	kilo Dalton
MBP	maltose binding protein
2-ME	2-mercaptoethanol
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide, reduced form
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NP40	nonidet-P40 (nonionic detergent)
nt	nucleotide
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
RACE	rapid amplification of cDNA ends
RbCl	rubidium chloride

RLM-RACE	RNA ligase-mediated rapid amplification of cDNA ends
RNase	ribonuclease
RT	reverse transcriptase
SAM	<i>S</i> -adenosyl methionine
SDS	sodium dodecyl sulfate
SHMT	serinehydroxymethyl transferase
SI	short isoform
SOE	splice overlap extension
TAP	tobacco acid pyrophosphatase
TBS	Tris-buffered saline
TCA	trichloroacetic acid
TEV	tobacco etch virus
THF	tetrahydrofolate
TOPO	topoisomerase I

APPENDIX II

Materials

Material	Manufacturer
Agarose	Invitrogen Life Technologies™ (Carlsbad, CA)
Ampicillin	Sigma-Aldrich Co. (St. Louis, MO)
Anti-Bcl-x _{S/L} antibodies	Santa Cruz Biotechnology (Santa Cruz, CA)
Anti-COXI antibodies	Molecular Probes (Eugene, OR)
Anti-Hsp60 antibodies	Stressgen Biotechnologies (San Diego, CA)
Anti-MnSOD antibodies	Stressgen Biotechnologies
Anti-porin antibodies	Calbiochem (San Diego, CA)
Anti-V5 antibodies	Invitrogen Life Technologies
BSA	Sigma-Aldrich Co.
Cell culture dishes (Nunc)	VWR (West Chester, PA)
Centriprep® YM-10	Millipore Corp. (Bedford, MA)
Chloramphenicol	Sigma-Aldrich Co.
Difco media components	VWR
Digitonin	Sigma-Aldrich Co.
dNTP	Invitrogen Life Technologies
ECL Western Blotting Reagents	Amersham Biosciences Corp. (Piscataway, NJ)
EDTA	Sigma-Aldrich Co.
Fast Link™ DNA Ligation Kit	Epicentre® Technologies (Madison, WI)
Fetal Bovine Serum	Atlanta Biologicals (Lawrenceville, GA)
FirstChoice™ RLM-RACE Kit	Ambion, Inc. (Austin, TX)
Geneticin® (G418 Sulfate)	EMD Biosciences (La Jolla, CA)
Glutamax	GIBCO BRL (Gaithersburg, MD)

HRP-goat anti-mouse or anti-rabbit IgG _γ	Zymed Laboratories Inc. (San Francisco, CA)
HyQ α-minimal eagle's medium	Hyclone (Logan, UT)
Imidazole	Sigma-Aldrich Co.
IPTG	Research Products International Corp. (Mt. Prospect, IL)
KOD Hot Start DNA Polymerase	Novagen (Madison, WI)
MasterAmp TM <i>Tfl</i> DNA Polymerase	Epicentre Technologies
2-ME	Sigma-Aldrich Co.
Methanol	Fisher Scientific Co. (Fair Lawn, NJ)
Nitrocellulose membrane	Schleicher and Schuell Bioscience, Inc. (Keene, NH)
PAGE supplies	Bio-Rad Laboratories, Inc. (Hercules, CA)
pBluescript KS (+/-)	Stratagene [®] (La Jolla, CA)
pcDNA3.1 [©] Directional TOPO [®] Expression Kit	Invitrogen Life Technologies
[α- ³² P]dATP	PerkinElmer Life Sciences (Wellesley, MA)
Penicillin/Streptomycin	GIBCO BRL (Gaithersburg, MD)
pET 22b	Novagen
<i>Pfu</i> DNA Polymerase	Stratagene
pGEM T-Easy	Promega (Madison, WI)
PMSF	Sigma-Aldrich Co.
Primers	Integrated DNA Technologies (Coralville, IA)
Protein A Sepharose beads	Pierce (Rockford, IL)
Protein assay dye reagent	Bio-Rad Laboratories, Inc.
QIAGEN DNA purification kits	QIAGEN Inc. (Valencia, CA)
QuikChange [®] Site-Directed Mutagenesis Kit	Stratagene

Restriction endonucleases	New England Biolabs (Ipswich, MA)
Rosetta 2(DE3)pLysS (<i>E. coli</i> strain)	Novagen
Silver staining reagents	Bio-Rad Laboratories, Inc.
Sodium butyrate	Sigma-Aldrich Co.
Strip-EZ™ PCR Kit	Ambion, Inc.
TALON Metal Affinity Resin	BD Biosciences (San Jose, CA)
<i>Taq</i> DNA Polymerase	Applied Biosystems (Foster City, CA)
Triton X-100	Beckman® Analytical Chemicals
Trypsin	Cellgro (Herndorn, VA)
XL1-Blue (<i>E. coli</i> strain)	Stratagene

APPENDIX III

Bacterial Strains and Plasmids

Bacterial Strains

Strain	Genotype	Application
XL1-Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [F' <i>proAB lacI^qΔM15Tn10</i> (Tet ^r)]	Used for transformation and plasmid DNA isolation. Source: Stratagene
Rosetta2 (DE3)pLysS	F ⁻ <i>ompT hsdS_B</i> (r _B ⁻ , m _B ⁻) <i>gal dcm</i> (DE3) pLysS RARE2 (Cam ^R); a BL21 derivative carrying bacteriophage DE3 with the T7 RNA Polymerase gene under the control of lacUV5, also contains pLysS plasmid with genes for T7 lysozyme and tRNA genes for rare codons in <i>E. coli</i>	Used for overexpression of pMal system plasmid. Source: Novagen

Plasmids

Name of Plasmid

Description

Chapter 2

DKFZp586G1517

Partial cDNA clone of human mitochondrial C₁-THF synthase constructed by the German Genome Project (RZPD German Research Center for Genome Research; Wiemann *et al.*, 2001)

dJ44A20

Genomic artificial P1 chromosome clone (Sanger Centre, Cambridge, UK)

pGEM T-Easy

Used to subclone exon 1 from dJ44A20 (Promega)

pcDNA3.1D/V5-His-TOPO

Used for directional TOPO cloning of full-length human mitochondrial C₁-THF synthase cDNA generated by SOE-PCR (Invitrogen)

pcDNA3.1-humito

V5 epitope-tagged full-length cDNA clone of human mitochondrial C₁-THF synthase in pcDNA3.1D/V5-His-TOPO vector

pBluescriptKS(+/-)

Used for site-directed mutagenesis and also for cloning 5' and 3' RACE PCR products (chapters 3 & 4) for sequence analysis (Stratagene)

pBluescriptKS(+/-)/

pcDNA3.1-humito (816-bp)

Clone of an 816-bp DNA fragment of pcDNA3.1-humito in pBluescriptKS(+/-)

Chapter 3

pUC13/HS230

Clone of 230-bp fragment of the human cytoplasmic C₁-THF synthase cDNA (obtained from Dr. R. E. MacKenzie, McGill University, Hum *et al.*, 1988)

Chapter 4

pET22b

E. coli vector for overexpression of recombinant proteins (Novagen)

pET22b-short

pET vector for expression of short isoform of human mitochondrial C₁-THF synthase in *E. coli*

pMal-c2x H10TEV

E. coli vector for overexpression of recombinant proteins (obtained from Dr. John Tesmer (Kristelly *et al.*, 2004))

pMal-short isoform

pMal vector for expression of short isoform of human mitochondrial C₁-THF synthase in *E. coli*

APPENDIX IV

Protocols

Protocol 1: Standard Polymerase Chain Reaction

Most PCR reactions were performed using the *Taq* DNA Polymerase (Applied Biosystems). MasterAmp™ *Tfl* DNA Polymerase (Epicentre) (lacks 3' to 5' proofreading nuclease activity) or KOD Hot Start DNA Polymerase (Novagen) was used for GC-rich templates. Addition of betaine (1 M final concentration) improved amplification with the latter. All other cloning procedures employed *Pfu* DNA Polymerase (Stratagene), which has 3' to 5' proofreading nuclease activity.

For GC-rich plasmid DNA templates:

1. Assemble:
 - 5 µl 10X PCR buffer (supplied with enzyme)
 - 5 µl dNTPs (0.2 mM final concentration, supplied with enzyme)
 - 2 µl MgSO₄ (1 mM final concentration, supplied with enzyme)
 - 10 µl template DNA (1 ng/µl)
 - 1.5 µl 10 µM 5' primer
 - 1.5 µl 10 µM 3' primer
 - 24 µl sterile ddH₂O
 - 1 µl KOD Hot Start DNA Polymerase (1 U/µl)
 - 50 µl total volume; overlay with two drops of mineral oil
2. Perform amplification in a thermal cycler:
 - a) Activate 94°C, 2 min.; 1 cycle

- b) Denature 94°C, 15 sec.; Anneal at primer T_m -5°C, 30 sec.; extend at 68°C 1min. per 1 kb; 30 cycles

OR

1. Assemble:

2.5 µl 20X *Tfl* buffer (supplied with enzyme)
4 µl 2.5 mM dNTPs
3 µl 25 mM MgCl₂ (supplied with enzyme)
15 µl 10X PCR Enhancer (supplied with enzyme)
10 µl template DNA (1 ng/µl)
1 µl 10 µM 5' primer
1 µl 10 µM 3' primer
13 µl sterile ddH₂O
0.5 µl *Tfl* DNA Polymerase (1 U/µl)
50 µl total volume; overlay with two drops of mineral oil

2. Perform amplification in a thermal cycler:

- a) Activate 94°C, 3 min.; 1 cycle
b) Denature 94°C, 30 sec.; Anneal at primer T_m -5°C, 30 sec.; extend at 72°C 1min. per 1 kb; 30 cycles
c) Extend 72°C, 7 min.; 1 cycle

For non-GC rich plasmid DNA templates:

1. Assemble:

10 μ l 10X PCR buffer (supplied with enzyme)

8 μ l 2.5 mM dNTPs

10 μ l template DNA (1 ng/ μ l)

2 μ l 10 μ M 5' primer

2 μ l 10 μ M 3' primer

0.5 μ l *Taq* DNA Polymerase (5 U/ μ l) or 2 μ l *Pfu* DNA Polymerase (2.5 U/ μ l)

Make upto 100 μ l with sterile ddH₂O; overlay with 2 drops of mineral oil

2. Perform amplification in a thermal cycler:

a) Activate 94°C, 3 min.; 1 cycle

b) Denature 94°C, 30 sec.; Anneal at primer T_m-5°C, 30 sec.; extend at 72°C 1min. per 1 kb; 30 cycles

c) Extend at 72°C, 10 min.; 1 cycle

Protocol 2: Transformation of RbCl-Competent *E. coli* Cells

(Hanahan, 1985)

Solutions:

1. **TFB1:** 30 mM KOAc, 100 mM RbCl, 10 mM CaCl₂, 50 mM MnCl₂, 15% (w/v) glycerol, adjust pH to 5.8 with acetic acid and filter through a 0.45 µm filter.
2. **TFB2:** 10 mM MOPS, 10 mM RbCl, 75 mM CaCl₂, 15% (w/v) glycerol, adjust pH to 6.5 with KOH and filter sterilize.

Protocol:

A. To make RbCl-Competent cells:

1. Inoculate 2.5 ml YT (0.5 % yeast extract, 0.8 % Tryptone, and 0.5 % NaCl) medium with a single colony of *E. coli* strain XL1-Blue and grow overnight at 37°C with shaking.
2. Inoculate 250 ml YT + 20 mM MgSO₄ using the overnight culture and grow to an OD₆₀₀ of 0.4-0.6.
3. Harvest the cells in a sterile tube by centrifuging at 5000 X g for 5 min. at 4°C.
4. Resuspend the pellet gently in 100 ml of TFB1, followed by incubation on ice for 5 min.
5. Repeat centrifugation at 5000 X g for 5 min. at 4°C.
6. Resuspend the pellet gently in 10 ml of TFB2, followed by incubation on ice for 10-60 min.
7. Make aliquots of 50 µl, freeze them in liquid nitrogen, and store at -70°C.

B. To transform RbCl-competent cells:

1. Thaw an aliquot of competent cells on ice.
2. Add 100-500 ng of plasmid DNA and mix well.
3. Incubate on ice for 60 min.
4. Heat shock the cells at 42°C for 45 sec.
5. Add to a sterile tube containing 150 µl YT and incubate at 37°C for 20 min. with shaking.
6. Spread the cells on a YT/agar (YT + 2% agar) plate containing the appropriate antibiotic for selection.

Protocol 3: TOPO Cloning and One Shot TOP10 Chemical Transformation

TOPO cloning was performed as per pcDNA3.1[©] Directional TOPO[®] Expression kit (Invitrogen) protocol. It was employed to clone the full-length human mitochondrial C₁-THF synthase cDNA constructed by SOE-PCR into the mammalian expression vector pcDNA3.1D/V5-His-TOPO. The TOPO-cloned DNA was transformed into *E. coli* by One Shot[®] TOP10 chemical transformation (Invitrogen). The kit provided all the necessary solutions and reagents.

Protocol:

1. Mix 2 µl full-length cDNA with 1 µl salt solution (1.2 M NaCl, 0.06 M MgCl₂) in a 0.6 ml microfuge tube. Make up the volume to 5 µl with sterile ddH₂O.
2. Add 1 µl TOPO[®] vector, mix gently and incubate at room temperature for 5 min.
3. Keep the TOPO[®] reaction on ice. Transfer 2 µl of TOPO[®] reaction into a tube containing One Shot Chemically Competent *E. coli* and mix gently by tapping the tube at the bottom.
4. Incubate on ice for 10 min.
5. Heat shock at 42°C for 30 sec. Keep the tube on ice.
6. Add 250 µl of SOC medium (2 % tryptone, 0.5 % yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM Mg SO₄, 20 mM glucose) to the cells and rotate (200 rpm) at 37°C for 1 h.
7. Spread 200 µl of the transformation reaction on a YT/agar (0.5 % yeast extract, 0.8 % Tryptone, 0.5 % NaCl, and 2% agar) plate containing 50 µg/ml ampicillin. Incubate overnight at 37°C.

Protocol 4: 5'- and 3'-RNA Ligase-Mediated Rapid Amplification of cDNA Ends (RLM-RACE)

5'-RACE

10 µg human placental total RNA (Ambion Inc.) was used in a “standard” reaction for 5'-RACE as per Ambion's FirstChoice RLM-RACE kit protocol. The kit provided all the necessary solutions and reagents. The sample can be stored at -20°C after Calf Intestinal Phosphatase (CIP) treatment and extraction or Tobacco acid pyrophosphatase (TAP) treatment or RNA adapter ligation or reverse transcription before proceeding to the next step. See section 3.2.2 for other details.

A. RNA Processing

1. Calf Intestinal Phosphatase (CIP) treatment

Assemble:

10 µl human placental total RNA (1µg/µl)

2 µl 10X CIP buffer

2 µl CIP

6 µl nuclease-free water

20 µl total volume

Incubate at 37°C for 1 h. Terminate CIP reaction, and perform extraction and ethanol precipitation as in section 3.2.2c before TAP treatment.

2. Tobacco Acid Pyrophosphatase (TAP) treatment

Assemble:

5 µl CIP treated RNA

1 µl 10X TAP buffer

2 µl TAP

2 µl nuclease-free water

10 µl total volume

Incubate at 37°C for 1 h. followed by RNA adapter ligation.

3. RNA adapter ligation

Assemble:

2 µl CIP/TAP-treated RNA or 1 µl minus-TAP RNA control

1 µl RNA adapter

1 µl 10X RNA Ligase buffer (warm the buffer tube by rolling between hands)

1 µl T4 RNA Ligase (5 U/µl)

Make upto 10 µl with nuclease-free water

Incubate at 37°C for 1 h. followed by reverse transcription.

B. Reverse Transcription

Assemble:

2 µl ligated RNA or minus-TAP ligation control

4 µl dNTP mix

2 µl random decamers

2 µl 10X RT-PCR buffer

1 µl RNase inhibitor

1 µl MMLV Reverse Transcriptase (RT)

8 µl nuclease-free water

20 µl total volume

Incubate at 42°C for 1 h. Perform nested PCR with *Tfl* DNA Polymerase (see protocol 1) using 1 µl RT reaction for outer PCR and 1 µl outer PCR product for inner PCR as in section 3.2.2e. Minus-TAP and minus-template controls were included.

3'-RACE

1 µg human placental total RNA (Ambion Inc.) was used for 3'-RACE as per Ambion's FirstChoice RLM-RACE kit protocol. The kit provided all the necessary solutions and reagents.

Reverse Transcription

Assemble:

1 µl human placental total RNA (1µg/µl)

4 µl dNTP mix

2 µl 3'-RACE adapter

2 µl 10X RT-PCR buffer

1 µl RNase inhibitor

1 µl MMLV Reverse Transcriptase (RT)

8 µl nuclease-free water

20 µl total volume

Incubate at 42°C for 1 h. Perform nested PCR with Taq DNA Polymerase (see protocol 1) using 1 µl RT reaction for outer PCR and 1 µl outer PCR product for inner PCR as in section 4.2.1. A minus template control was included.

Nested PCR (5'- and 3'-RACE)

Nested PCR ("outer" and "inner" PCR): Assemble the reaction for the respective DNA Polymerase.

Perform amplification in a thermal cycler:

- a) Activate 94°C, 3 min.; 1 cycle
- b) Denature 94°C, 30 sec.; Anneal at 60°C (5'-RACE) or 59°C (3'-RACE), 30 sec.; extend at 72°C 1min. per 1 kb; 35 cycles
- c) Extend at 72°C, 7 min.; 1 cycle

Protocol 5: CHO Cell Culture

Solutions:

1. **Complete medium:** 900 ml HyQ α -minimal Eagle's medium + 100 ml fetal bovine serum (10% (v/v) final) + 20 ml of 12.5 mM glutamax (0.25 mM final) + 10 ml of 10,000 μ g/ml penicillin/streptomycin (100 μ g/ml final), store at 4°C.
2. **Phosphate Buffered Saline (PBS):** 10 mM phosphate buffer (pH 7.4), and 140 mM NaCl.
3. **Geneticin (G418 Sulfate) Stock (100 mg/ml):** Add 10 ml PBS into 1 g G418, filter through a 0.45 μ m filter, store in aliquots at -20°C.
4. **1X Trypsin (0.25%):** Dilute 5 ml of 2.5% (w/v) trypsin with 45 ml PBS, add 50 μ l 0.5 M EDTA (pH 8.0)), store at 4°C.
5. **5% Dimethyl Sulfoxide (DMSO):** Add 9.5 ml complete medium into 0.5 ml DMSO.

Protocol:

A. To propagate cells:

1. Warm complete medium and 1X trypsin to 37°C.
2. Thaw a frozen stock of CHO cells quickly by vigorous shaking at 37°C.
3. Place 14 ml of complete medium in a 100-mm diameter culture dish (P100) and pipette the thawed cells into it.
4. Incubate the dish in a 37°C incubator in an atmosphere of 5% CO₂ overnight.
5. Aspirate the old medium, add 10 ml fresh medium and incubate at 37°C in an atmosphere of 5% CO₂ until cells are 100% confluent (CHO cells usually need ~48 hours to become 100% confluent).

6. Aspirate the old medium, wash the cells carefully 1X with 5 ml PBS and trypsinize the cells by adding 2 ml 1X trypsin. Incubate at 37°C in an atmosphere of 5% CO₂ for 5 min.
7. Stop trypsinization by adding 8 ml complete medium. Distribute cells into 4 dishes by diluting trypsin-treated cells:complete medium in a ratio of 1: 4 (v/v) (cells from 1 P100 dish are placed into 4 P100 dishes). Add 80 µl G 418 stock/10 ml medium (0.8 mg/ml final) for selection of transfectants. This is passage 1 (P1). Incubate dishes as above.

Note: Use 150-mm diameter dishes (P150) to yield enough cells for isolation of mitochondria (see protocol 6). Cells from one P100 dish should all go into one P150 dish.

B. To make frozen stock:

1. When 100% confluent, wash cells with PBS (1 X 5ml) at room temperature and trypsinize as above.
2. Pool the dislodged cells in a centrifuge tube and harvest the cells by centrifugation at 1000 X g for 5 min.
3. Remove supernatant, add 1ml 5% DMSO in complete medium, mix and add to a cryovial. Allow slow freezing in an atmosphere of isopropanol at -70°C. Store in liquid nitrogen eventually.

Protocol 6: Isolation of mitochondria from CHO Cells

(Rickwood *et al.*, 1987; Pallotti and Lenaz, 2001)

Isolation of CHO cell mitochondria was performed as described for tissue culture cells (Rickwood *et al.*, 1987).

Solutions:

1. **Phosphate Buffered Saline (PBS):** see protocol 5.
2. **Homogenization solution (HMS):** Make a solution of 0.25 M sucrose, 1 mM EGTA, 10 mM HEPES, adjust pH to 7.4 with NaOH at room temperature and dissolve 0.5% (w/v) BSA using a stir bar at 4°C.
3. **HMS without BSA:** Prepare same as above except for adding BSA.

Protocol:

1. Plate CHO cells transfected with pcDNA3.1-humito on P150 dishes and culture as in protocol 5.
2. When 100% confluent, wash cells with PBS (1 X 5ml) at room temperature and trypsinize (see protocol 5).
3. Pool the dislodged cells in a centrifuge tube and count the cells using a hemocytometer using a 1:10 dilution in PBS.
4. Harvest the cells by centrifugation at 750 X g for 5 min. at room temperature.
5. Wash the cells 1 X with PBS and 1 X with HMS (4 ml/0.5 X 10⁸ cells) by centrifuging at 750 X g for 5 min. at room temperature. All the following steps are done on ice.
6. Resuspend the cells in the same volume of HMS as above and transfer to a tight-fitting glass-teflon Thomas homogenizer (pre-cooled on ice), and homogenize by 20 up-and-down manual strokes (Pallotti and Lenaz, 2001).

7. Centrifuge the homogenate at 1500 X g for 10 min. to pellet nuclei and any unbroken cells. Remove the supernatant carefully into another centrifuge tube and store on ice.
8. Resuspend the pellet from step 6 in the same volume of HMS as in step 4, repeat homogenization and centrifuge at 1500 X g for 10 min.
9. Combine the supernatant with the first supernatant, centrifuge at 1500 X g for 10 min. and discard any pellet. The final supernatant is the post-nuclear supernatant fraction.
10. Centrifuge the final supernatant at 10,000 X g for 10 min. and store any pellet on ice.
11. Repeat centrifugation as above; the supernatant is the cytosolic fraction.
12. Mitochondrial pellets from steps 9 and 10 are combined by resuspending (use a test tube filled with ice) the pellet in 1 ml each of HMS, bring volume to 10 ml, and centrifuge at 10,000 X g for 10 min. Repeat the rinsing step 2 X.
13. Resuspend the final pellet in 1 ml HMS without BSA to give the mitochondrial fraction.

Note: A Sorvall SS-34 rotor was used for all centrifugations from step 6 onwards.

Protocol 7: Isolation of Mitochondria from Rat Spleen

(Pedersen *et al.*, 1978; Rickwood *et al.*, 1987)

Isolation of rat spleen mitochondria was performed using the same procedure described for rat liver mitochondria (Pedersen *et al.*, 1978).

Solutions:

1. **H-Medium:** Prepare 0.3 M sucrose with 1 mM EGTA, 5 mM MOPS, 5 mM KH_2PO_4 , adjust pH to 7.4 with NaOH at room temperature and dissolve 0.1% (w/v) BSA using a stir bar at 4°C (Rickwood *et al.*, 1987).
2. **H-Medium without BSA:** Prepare as above except for adding BSA.

Protocol:

7. Fast two 8 weeks old Sprague-Dawley male rats overnight, euthanize, remove spleens (~1 g each), and placed in a beaker containing 5 ml ice-cold H-medium.
2. While keeping in H-medium, mince the spleens with scissors and wash until all blood is removed.
3. Transfer the tissue to a tight-fitting glass teflon Thomas homogenizer (pre-cooled on ice) containing 5 ml (20% w/v) ice-cold H-medium and homogenize by 4 up-and-down strokes of the Teflon pestle attached to a drill press operating at 870 rpm.
4. Dilute the homogenate to 18 ml, transfer to a centrifuge tube, and centrifuge at 660 X g for 15 min.
5. Remove the supernatant carefully and centrifuge at 7100 X g for 15 min. to pellet the mitochondria.
6. Remove supernatant and fluffy layer on top of the mitochondrial pellet carefully to yield the cytoplasmic fraction.

7. Resuspend pellet in 1 ml H-medium using a test tube filled with ice, bring volume to 10 ml, and centrifuge at 9770 X g for 15 min. Repeat the rinsing step 1 X using 5 ml H-medium.
8. Resuspend the pellet in 500 μ l H-medium without BSA to yield the mitochondrial fraction (usually this will give a mitochondrial protein concentration of $\sim$ 15 mg/ml).

Note: A Sorvall SS-34 rotor was used for all centrifugations.

Protocol 8: Isolation of Mitochondria from Human Placenta

(from Dr. JoAnn Johnson, University of Texas at Austin; Gray and Wong, 1992)

Human placenta was kindly provided by St. David's Medical Center (Austin, TX) and given to us by Dr. JoAnn Johnson (University of Texas, Austin, TX). It was washed in H-medium (see protocol 7), frozen in liquid nitrogen, and stored at -70°C until use.

Solutions:

1. **250 mM sucrose**
2. **Buffer A:** 250 mM sucrose, 1mM EDTA, 50 mM Tris-HCl (pH 7.5)
3. **Buffer B:** 250 mM sucrose, 1 mM EDTA, 10 mM Tris-HCl (pH 7.5)

Protocol:

1. Thaw placenta and weigh a piece of about 5g.
2. Thaw completely in chilled 250 mM sucrose kept on ice.
3. Remove any connective tissue and wash 3 X in buffer A.
4. Dice with scissors while keeping in 12.5 ml buffer A (2.5 X the wet weight of the tissue) and transfer to two 15 ml corex tubes.
5. Homogenize the tissue in a Tekmar tissumizer at high speed for 5 X 5 sec. each, incubating on ice for 30 sec. after each cycle. Pool the homogenates.
6. Centrifuge the pooled homogenate at 960 X g for 15 min., pour the supernatant through 2 layers of cheese cloth and centrifuge at 17,200 X g for 15 min.
7. Save the pellet on ice and centrifuge the supernatant at 17,200 X g for another 15 min.
8. The supernatant is the cytoplasmic fraction. Combine the pellet with the first pellet using 10 ml of buffer B and centrifuge at 17,200 X g for 15 min.

9. Discard the supernatant. Wash the pellet 2 X with buffer B and resuspend in 750 μ l buffer B to yield the mitochondrial fraction (usually this will give a mitochondrial protein concentration of $\sim$ 10 mg/ml).

Note: A Beckman JA20 rotor was used for all centrifugations.

Protocol 9: Immunoblotting
(Tibbetts and Appling, 2000)

Solutions:

1. 20X Transfer Buffer (200 ml)

0.2 M Tris base (4.84 g)

2 M glycine (30.03 g)

200 ml of 20X transfer buffer is mixed with 400 ml methanol and 3400 ml ddH₂O to prepare 1X transfer buffer

2. 20X TBS (100 ml)

0.2 M Tris base (2.42 g)

3 M NaCl (17.53 g)

Add 80 ml ddH₂O and adjust the pH to 8.0 with HCl

Make up the volume to 100 ml (can be autoclaved)

20X TBS is diluted 20-fold in ddH₂O to prepare 1X TBS (10 mM Tris-Cl, 150 mM NaCl (pH 8.0))

Prepare 1X TBST by adding Tween-20 to 1X TBS to a final concentration of 0.05%

Protocol:

1. Run the protein samples and Bio-Rad precision protein standards on an SDS-PAGE gel.
2. Place the gel in 1X transfer buffer for a few min.
3. Cut a piece of nitrocellulose membrane of the same gel size and cut two pieces of Whatman 3 MM filter paper slightly larger than the nitrocellulose membrane.
4. Wet the nitrocellulose membrane in 1X transfer buffer and carefully place on

top of the gel without trapping any air bubbles in between. Make a sandwich with the gel and nitrocellulose membrane kept between two pieces of filter paper, and assemble the electroblot apparatus.

- Note: Orient the gel holder with the gel closer to the cathode (black) and the membrane closer to the anode (red).
5. Electroblot in the cold room at 250 mA (~120 V) for 75 min. Make sure to keep the buffer stirring with a stir bar.
 6. Disassemble electroblot apparatus and wash the nitrocellulose membrane with ddH₂O (3 X, 5 min.).
 7. Block binding sites on the membrane with 2% milk powder (Sanalac) in 1X TBS for 1 h with shaking.
 8. Remove the blocking solution and add primary antibody appropriately diluted in 1X TBS containing 1% dry milk. (The antibodies used are mouse anti-V5 antibody (see “Experimental Procedures” for dilutions used; Invitrogen), rabbit anti-mito-C₁-THF synthase antibody (1:1000 dilution; see Chapter 4), rabbit anti-porin (1:2000 dilution, 0.5 µg/ml; Calbiochem), rabbit anti-Bcl-x_{S/L} (S-18) (1:1000 dilution, 0.2 µg/ml; Santa Cruz Biotechnology), mouse anti-COXI (1:1000 dilution, 1 µg/ml; Molecular Probes), rabbit anti-Hsp60 (1:2000 dilution, Stressgen Biotechnologies), or rabbit anti-MnSOD (1:2000 dilution, 0.5 µg/ml; Stressgen Biotechnologies). Incubate for 1 h with shaking.
 9. Wash the membrane with 1X TBST (3 X, 5 min).
 10. Add secondary antibody in a 1:2000 dilution in 1X TBST (10 µl horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibody (Zymed Laboratories Inc) in 20 ml 1X TBST). Incubate for 1 h with shaking.
 11. Wash the membrane with TBST (2 X, 5 min.).
 12. Wash the membrane with TBS (2 X, 5 min.).
 13. Rinse membrane with ddH₂O immediately before enhanced

chemiluminescence (ECL) detection.

14. In a dark room, mix the ECL Plus Western Blotting detection reagents (Amersham Biosciences) 1 and 2 in a ratio of 40:1, pipette onto the membrane, and incubate for 5 min.
15. Remove the excess reagents and place the membrane on a transparency sheet.
16. Visualize the signals using a Kodak Image Station 2000R (Kodak).

Protocol 10

Immunoprecipitation

CHO cells transfected with pcDNA3.1-humito were used to isolate mitochondria for immunoprecipitation. Mitochondria from untransfected cells were used as a control.

Solutions:

1. **Sodium butyrate:** 500 mM sodium butyrate (pH 7.0), sterilize using a 0.45 μ m filter, store at 4°C.
2. **NP-40 Cell Lysis Buffer:** 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40
3. **75 mM PMSF in 100% isopropanol**
4. **7X Protease Inhibitor Cocktail:** Resuspend 1 Complete Mini EDTA-free protease inhibitor cocktail tablet (Roche) in 1.5 ml ddH₂O.
5. **Protein A beads slurry** (Pierce)

Protocol:

A. Preparation of Mitochondrial Lysate

1. CHO cells were cultured as described in protocol 5.
2. When confluent, add sodium butyrate to a final concentration of 10 mM and allow to grow for another 20 h.
3. Harvest cells, and isolate mitochondria as described in protocol 6. Perform a Bradford assay to determine protein concentration.
4. Resuspend mitochondria in 1 ml cold NP-40 cell lysis buffer (500 μ l/mg of mitochondrial protein) containing 1mM PMSF and 1X protease inhibitor cocktail. Mix by vortexing gently.
5. Incubate on ice for 30 min, with occasional mixing.

6. Centrifuge at 10,000 X g for 10 min. at 4°C. The supernatant (mitochondrial lysate) was used for immunoprecipitation. Lysate can be stored at -70°C until use.

B. Preclearing of Mitochondrial Lysate

1. Take 100 µl of Protein A beads slurry in an Eppendorf tube and add 900 µl cold lysis buffer. Centrifuge at 10,000 X g for 30 sec. and remove the supernatant. Repeat washing with 1 ml cold lysis buffer and resuspend the beads in 100 µl cold lysis buffer.
2. Add mitochondrial lysate to the resuspended beads and rotate at 4°C for 1 h.
3. Centrifuge at 10,000 X g for 10 min. at 4°C, collect the supernatant, and centrifuge again to remove any bead. The final supernatant is the precleared lysate.

C. Immunoprecipitation

1. Add 2 µl of anti-V5 antibody (1.3 mg/ml) to the precleared lysate and rotate at 4°C for 1 h.
2. Add 100 µl of washed Protein A beads slurry in cold lysis buffer (see B1) and rotate at 4°C for 1 h.
3. Centrifuge at 10,000 X g for 30 sec. at 4°C and discard the supernatant. Wash the beads 3 X with 1 ml of lysis buffer. Make sure that the supernatant is removed completely in the washes.
4. Add 100 µl 1X Laemmli sample buffer to the pelleted beads. Mix by vortexing and heat to 90°C for 10 min.
5. Centrifuge at 10,000 X g for 5 min. at 4°C, collect the supernatant, and run the sample on an SDS-polyacrylamide gel. Silver stain the gel to visualize the proteins as per Bio-Rad protocol.

Protocol 11: Bradford assay

(Bradford, 1976)

Bradford assay was performed to determine the protein concentration using a modification of the Bio-Rad protocol. A microtiter plate was used to perform the assay in a total volume of 200 μl with bovine serum albumin (BSA) as a standard. The standards were used in a range of 0.5-4.0 μg in a volume of 50 μl . 150 μl each of 1X protein assay dye reagent concentrate (Bio-Rad) was added to the standards and appropriately diluted samples to develop the color. The absorbance was measured at 595 nm after incubating at room temperature for 5 min.

Protocol 12: Antisera Production

Antisera specific for human mitochondrial C₁-THF synthase were produced at M.D. Anderson Cancer Center (Department of Veterinary Sciences, Bastrop, TX). The antigen used was the short isoform (SI) of human mitochondrial C₁-THF synthase. Purified SI was electrophoresed on a 12% SDS-polyacrylamide gel, Coomassie-stained, destained, and the band containing SI (35 kDa) (see Chapter 4; *Figure 4.6*) was cut out for use as antigen. Two rabbits (0501 and 0502) were used for antisera production. 500 µg each of SI was used for primary immunization (Inoculation #1) and 200 µg each was used for secondary and subsequent immunizations (Inoculations #2, #3 and #4). Antisera from rabbit 0501 (test bleed # 3) were used for all immunoblots in chapters 4 and 5.

DAY	DATE	PROCEDURE
01	6/14/05	Pre-bleed and Inoculation #1
14	6/28/05	Inoculation #2
28	7/12/05	Test bleed #1 and Inoculation #3
42	7/26/05	Test bleed #2
76	8/30/05	Inoculation #4
90	9/13/05	Test bleed #3
124	10/17/05	Exsanguination and final bleed (antisera) collected

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