A pseudogene or not a pseudogene: that is the question?

PhD Thesis

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Abstract

Dihydrofolate reductase (DHFR) is a folate enzyme which reduces dihydrofolate into tetrahydrofolate in the presence of NADPH. DHFR was previously thought to be the only enzyme capable of this reaction however this report will show that humans have a second dihydrofolate reductase enzyme encoded by the former pseudogene *DHFRL1* (dihydrofolate reductase like – 1). It has been demonstrated that the *DHFRL1* gene is expressed and shares some commonalities with DHFR. Recombinant DHFRL1 can complement a *DHFR* negative phenotype in both bacterial and mammalian cells. Enzyme kinetics shows that the K_m for NADPH is similar for both enzymes but DHFRL1 has a higher K_m for dihydrofolate when compared to DHFR, indicating a lower affinity for the substrate. Localization of DHFRL1, visualized using confocal microscopy, shows that DHFRL1 has a strong presence in the mitochondria. DHFRL1 has the ability to bind its own mRNA in the same translational auto-regulation method as DHFR; with both enzymes capable of replacing each other. Methotrexate (MTX), a potent inhibitor of DHFR, is known to disrupt this regulation mechanism. DHFRL1, which has a lower binding affinity for MTX, requires a higher concentration of the drug to disrupt the protein: RNA binding complex. A *DHFR*- like sequence (DHFRLS) was also found to be expressed in rat and mouse. Although missing a number of amino acids at both terminals, rodent DHFRLS protein does display some enzyme activity. Enzyme kinetics reveals that the K_m for both NADPH and dihydrofolate are much higher for DHFRLS when compared to DHFR, indicating a lower affinity for both the substrate and the co-factor. Localization studies for the rodent DHFRLS did not however reveal a presence in the mitochondria. The identification of a second dihydrofolate reductase enzyme in humans encoded by a previously unrecognized retrogene will have a major impact on previous research surrounding DHFR.

Table of Contents

Chapter 3 Assessment of enzyme activity of DHFRL1

Chapter 4 The role of DHFRL1 in regulating both itself and DHFR

Chapter 5 Sub-cellular localization of DHFRL1

Chapter 8 General Discussion

Abbreviations

APS	Ammonium persulfate
ATCC	American Type Culture Collection
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
CBS	Cystathionine Beta Synthase
CHO	Chinese Hamster Ovary
CVD	Cardiovascular Disease
DFE	Dietary Folate Equivalent
DHFR	Dihydrofolate reductase
DHFRL1	Dihydrofolate reductase – like 1
DHFRLS	Dihydrofolate reductase like sequence
DMEM	Dulbecco's Modified Eagle Medium
DPBS	Dulbecco's Phosphate Buffered Saline
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkullturen
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
ENCODE	Encyclopedia of DNA Elements
EST	Expressed Sequence Tag
FACS	Fluorescence Activated Cell Sorting

FBS	Fetal Bovine Serum
FPGS	Folypoly-γ-glutamate synthase
GFP	Green Fluorescent Protein
GST	Glutathione S Transferase
GUS	Beta Glucuronidase
HEK	Human Embryonic Kidney
IMDM	Iscove's Modified Dulbecco's Medium
IPTG	Isopropyl β – D- 1- thiogalactopyranoside
LB	Luria Broth
LDS	Lithium Dodecyl Sulphate
MFT	Mitochondrial folate transporter
MTHFD1	Methylenetetrahydrofolate dehydrogenase methenyl tetrahydrofolate cyclohydrolase formyltetrahydrofolate synthetase
MTHFR	Methylenetetrahydrofolate reductase
MTRR	Methionine synthase reductase
MS	Methionine synthase
MTX	Methotrexate
NCBI	National Centre for Biotechnology Information
NTD	Neural Tube Defect
ORF	Open Reading Frame
PBS	Phosphate Buffered Saline

PCR	Polymerase Chain Reaction
PI	Propidium Iodine
RDA	Recommended Dietary Allowance
REMSA	RNA Electrophoretic Mobility Shift Assay
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
RT-qPCR	Reverse Transcriptase Quantitative Polymerase Chain Reaction
SAM	S-adenosylmethione
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SHMT	Serine hydroxymethyltransferase
SNP	Single Nucleotide Polymorhism
SUMO	Small ubiquitin like modifier protein
TBE	Tris Borate Ethylenediaminetetraacetic acid
TBS	Tris Buffered Saline
TEV	Tobacco Etch Virus
TMB	Tetramethylbendzidine
TS	Thymidylate Synthase

CHAPTER 1

Introduction

1.1 Overview

Folate mediated one-carbon metabolism is an essential biochemical pathway that has been extensively studied since the discovery of folic acid in the 1940's, and continues to be the focus of extensive research today. One-carbon metabolism has two important functions in the cell; 1) *de novo* nucleotide synthesis required for DNA replication and repair and 2) providing methyl donors for all cellular methylation reactions. [1] Folate is not produced endogenously within the cell and must be sourced through the diet, either in its natural form or the synthetic form of folic acid. An essential reaction of one-carbon metabolism is the reduction of dihydrofolate into tetrahydrofolate, a reaction carried out by dihydrofolate reductase (DHFR), tetrahydrofolate then feeds into the DNA cycle where it is used as a building block for purine and thymidylate synthesis . [2] DHFR is a highly conserved enzyme found in all species from protozoa to primates and carries out its function in both the cytoplasm and the nucleus of the cell.

In recent years a number of folate enzymes have been found to have different isoforms within the cell, particularly within different organelles within the cell. For example serine hydroxymethyltransferase (SHMT) has two isoforms, SHMT1 found in the cytoplasm and the nucleus and SHMT2 found in the mitochondria.[3] Methylenetetrahydrofolate dehydrogenase 1 (MTHFD1) carries out a number of essential reactions in the cytoplasm of the cell however in the mitochondria these reactions are carried out by a different isoform, MTHFD1L.[4]

It had previously been thought that there was only one functional dihydrofolate reductase enzyme within the cell; however with the increasing evidence for multiple isoforms of a number of essential folate enzymes, is DHFR the only enzyme capable of reducing dihydrofolate into tetrahydrofolate? Or is there a second previously unknown enzyme capable of this reaction in the cell?

The annotated *DHFR* gene family consists of one functional gene and four processed pseudogenes scattered across the genome. A processed pseudogene is formed through the reverse transcription of an mRNA molecule which is then re-inserted back into the genome. A classic characteristic of a processed pseudogene is the lack of introns. Once re-inserted into the genome the pseudogene usually acquires detrimental mutations which leave it non-functional.[5] The pseudogenes of *DHFR* were believed to fit the classic definition of a pseudogene and are non-

functional. While this may be true for three of these pseudogenes, the fourth annotated pseudogene, *DHFRL1*, has a confirmed mRNA transcript indicating a possible functionality within the cell.

Is *DHFRL1* a pseudogene as previously thought or is it a functional retrogene? Could *DHFRL1* encode a functional dihydrofolate reductase enzyme? What role would a second dihydrofolate reductase enzyme have within the cell? Where is the sub-cellular location of DHFRL1? How would the presence of a second dihydrofolate reductase enzyme affect our current understanding of DHFR? These questions are addressed in the following thesis

1.2 One-carbon folate mediated metabolism

Folate is an essential water soluble B vitamin that occurs naturally in green leafy vegetables such as spinach[6] and in its synthetic form, folic acid, in all fortified foods such as bread and breakfast cereals. For adults the Recommended Dietary Allowance (RDA) is 400μg/d of dietary folate equivalents (DFE), however for pregnant women the RDA is increased to 600μg/d DFE.[7] Cells convert folate and folic acid to the biologically active folate tetrahydrofolate. Tetrahydrofolate is then utilized by the cell in a process known as folate one-carbon metabolism [2] (Figure 1.1). One-carbon metabolism is an essential biochemical pathway that can be roughly dived into two cycles; the DNA replication cycle and the methylation cycle.[1] The DNA replication cycle, as the name suggests, uses tetrahydrofolate as a building block for the synthesis of purines and thymidylate essential for nucleic acid synthesis. The methylation cycle is responsible for the constant supply of methionine which when adenosylated to S-adenosyl methionine (Ado Met) becomes a methyl donor for many of the methylation reactions within the cell including DNA methylation which affects gene transcription.[8]

Humans do not generate folate endogenously and therefore it must be introduced into the system through the diet. Folate can only cross membranes in a monoglutamate form and are believed to cross the cellular membrane by three methods; 1) folate monoglutamates bind to and are internalized by folate receptors on the cell surface, 2) along with folate receptors folate monoglutamates can also enter the cell by reduced folate carrier mediated systems, 3) or alternatively folate monoglutamates can enter the cell by passive diffusion if the extra cellular concentration is higher than the intercellular concentration.[9] Once inside the cell the folate monoglutamate must be converted to polyglutamate form in order to be sequestered inside the cell. This reaction is carried out by the enzyme folypoly-γ-glutamate synthease (FPGS) which can add anything up to eight L-glutamic acid residues onto a folate monoglutamate substrate.[9]

1.2.1 Compartmentalization of one–carbon metabolism

Folate enzymes are present in every known organism and cell type. Within the cell folate mediated one–carbon metabolism is compartmentalized between the cytoplasm, mitochondria and to a lesser extent the nucleus. Figure 1.1 gives an overview of the compartmentalisation of one-carbon metabolism.

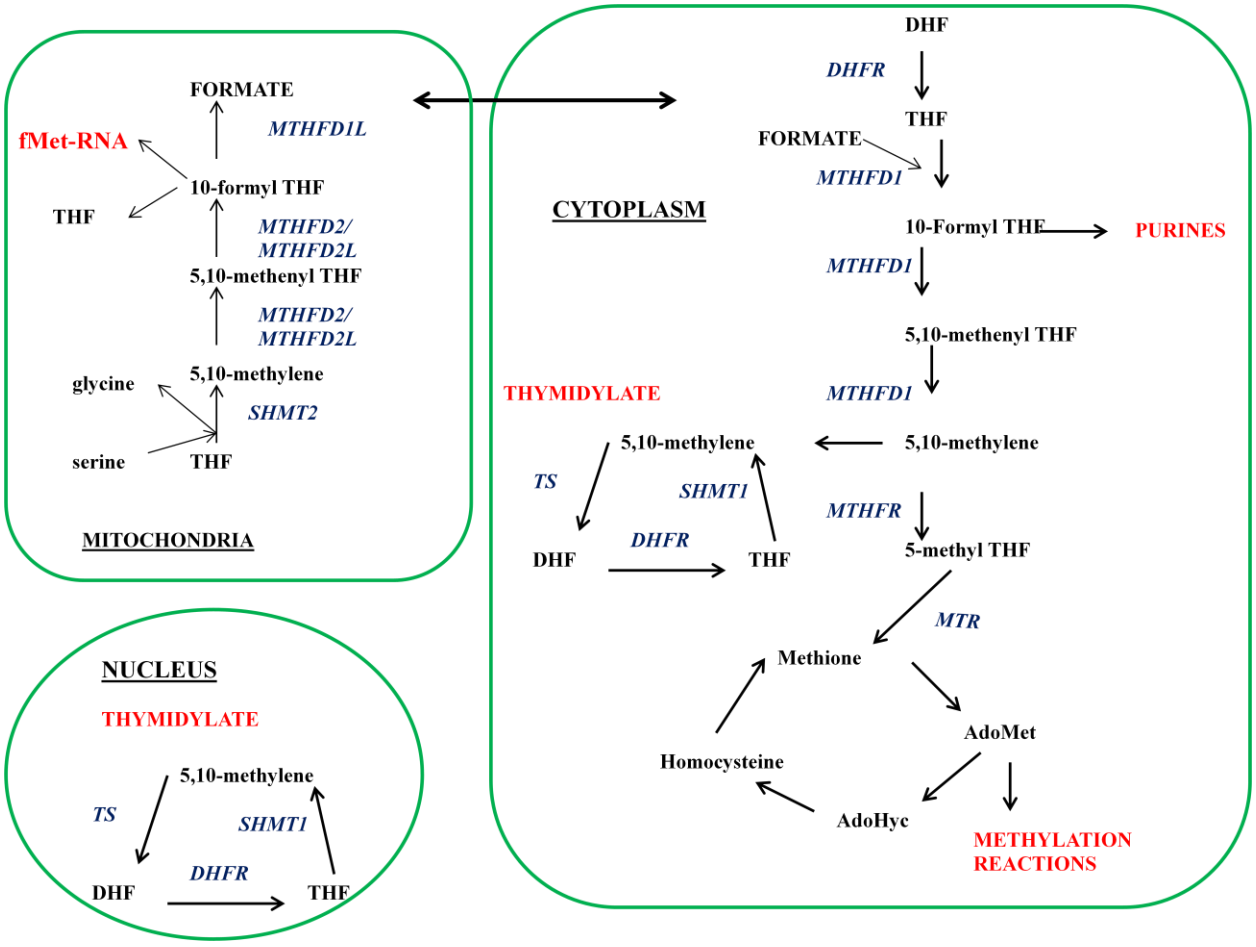

Figure 1.1 Overview of folate one-carbon metabolism. Image adapted from Tibbetts AS and Appling DR. (2010). *Annu. Rev. Nutr.* **30:**57-81. One-carbon metabolism is an essential biochemical pathway that is compartmentalized between the cytoplasm, mitochondria and nucleus. In the cytoplasm the pathway can be roughly divided into two cycles; the DNA cycle, supplying purines and thymidylate, and the methylation cycle. Mitochondrial folate metabolism produces Met-RNA an initiator RNA molecule required for protein synthesis and in the nucleus folate metabolism is mainly in the form of *de novo* thymidylate biosynthesis.

The majority of folate enzymes are found in the mitochondria and cytoplasm with the nucleus containing only low levels of folate.[10] Mitochondria contain almost 40% of total cellular folate however the folate polyglutamates found in the mitochondria differ from those found in the cytoplasm. [11] In the cytoplasm the majority of folate is found in the form of methyl-tetrahydrofolate (45%), in the mitochondria however 44% of total folate is found in the form of formyl-tetrahydrofolate. [11]

The pathways and functions of folate metabolism differ between the cytoplasm and the mitochondria. In the cytoplasm folate enzyme participate in the synthesis of purines and thymidylate as well as acting as methyl group donors. In the mitochondria folate enzymes are involved in the formylation of methionyl initiator RNA (MetRNA) which is needed for organelle protein synthesis. Mitochondrial folate metabolism is also a major pathway for serine and glycine catabolism.[10]

There are a number of mechanisms by which folate derivatives can enter the mitochondria. One such mechanism is carrier – mediated transport systems such as mitochondrial folate transporter (MFT) protein. However this system is not enough to supply the entire one-carbon requirements of the mitochondria.[12] The deficit of supply of one-carbon units by this mechanism is balanced out by the transport of one-carbon donors such as serine, glycine and formate between the cytoplasm and mitochondria.[13]

Despite the differences between cytoplasmic and mitochondrial one-carbon metabolism, the two pathways are not distinct from one another but are inter - dependent. There is significant evidence for the involvement of mitochondrial folate metabolism in the supply of cytoplasmic one-carbon units. An example of this co-dependence is the production and utilization of formate. Mitochondria metabolises formate some of which then exits the mitochondria and is utilized by the cytoplasmic folate pathway.[14] Experiments carried out using glycine requiring CHO mutants also support the inter – dependence of mitochondrial and cytoplasmic folate pathways, with these mutants revealing that mitochondrial serine hydroxymethyltransferase (SHMT) activity is essential for glycine metabolism in both the mitochondria and the cytoplasm.[15]

Folate metabolism in the nucleus mainly occurs in the form of *de novo* thymidylate synthesis. In the cytoplasm this pathway contains three enzymes; dihydrofolate reductase, thymidylate

synthase and serine hydroxymethyltransferase. These three enzymes also take part in *de novo* thymidylate synthesis the nucleus with all three enzymes undergoing sumoylation by the SUMO-1 protein to facilitate import into the nucleus during S phase of the cell cycle when DNA synthesis is at its peak.[16]

1.3 One-carbon metabolism and disease

As folate cannot be produced endogenously by the cell it is essential that a sufficient level of folate is obtained through diet. Severe folate deficiency has been linked to a wide spectrum of clinical diseases including megaloblastic anaemia. Megaloblasts are pre-cursors for erythrocytes and are the result of the failure of the red blood cell pre-cursors to divide normally. In cases where folate deficiency is present, the megaloblasts accumulate in the bone marrow diminishing cell division and also reducing the number of white blood cells and platelets.[17]

Moderate folate deficiency is a common environmental factor in a number of multi-factorial diseases including neural tube defects, cardiovascular disease, and cancer. The role of folate status in the development of these diseases is discussed in more detail below.

1.3.1 One-carbon metabolism and links to neural tube defects.

As folate metabolism is essential to the cell any impairment or deficiency can have severe consequences. One such disease linked with impaired one-carbon metabolism is neural tube defects (NTD). NTDs are among the most common congenital birth defects worldwide. NTD is an umbrella name for a group of central nervous system dysfunctions which come about when the neural tube, which is the embryonic precursor of the brain and spinal cord, fails to close during neurulation. This occurs very early in pregnancy usually around day 28.[18] NTDs are multi-factorial diseases caused by genetic factors, environmental factors and interactions between the two. Although numerous environmental and genetic factors contribute NTDs, accumulating evidence from decades of research demonstrate that folate status is a significant determinant of NTD risk.

The link between NTDs and folate deficiency was first proposed by Smithells *et al* in 1976 [19] who observed a decrease in several micronutrients, but especially folate, in the serum of women pregnant with a NTD affected child during the first trimester. Subsequent controlled folic acid

supplementation intervention trials conclusively showed that folic acid supplementation could reduce the risk of neural tube defects by up to 70%. [20] It is unclear how exactly folic acid supplementation prevents NTDs, however there are a number of hypothesis; 1) folic acid supplementation is acting directly to correct inadequate folate levels, 2) acting indirectly to overcome metabolic impairment either genetic or environmental or 3) folic acid supplementation may be exerting a positive protective effect via another unknown mechanism independent of folate metabolism.[21] Impairments in folate mediated one carbon metabolism can have a number of sources including SNPs in folate related genes, reduced folate status or secondary micronutrients deficiencies of nutrients that alter folate status such as other B vitamins. Figure 1.2 gives an overview of the possible links between folate status and neural tube defects.

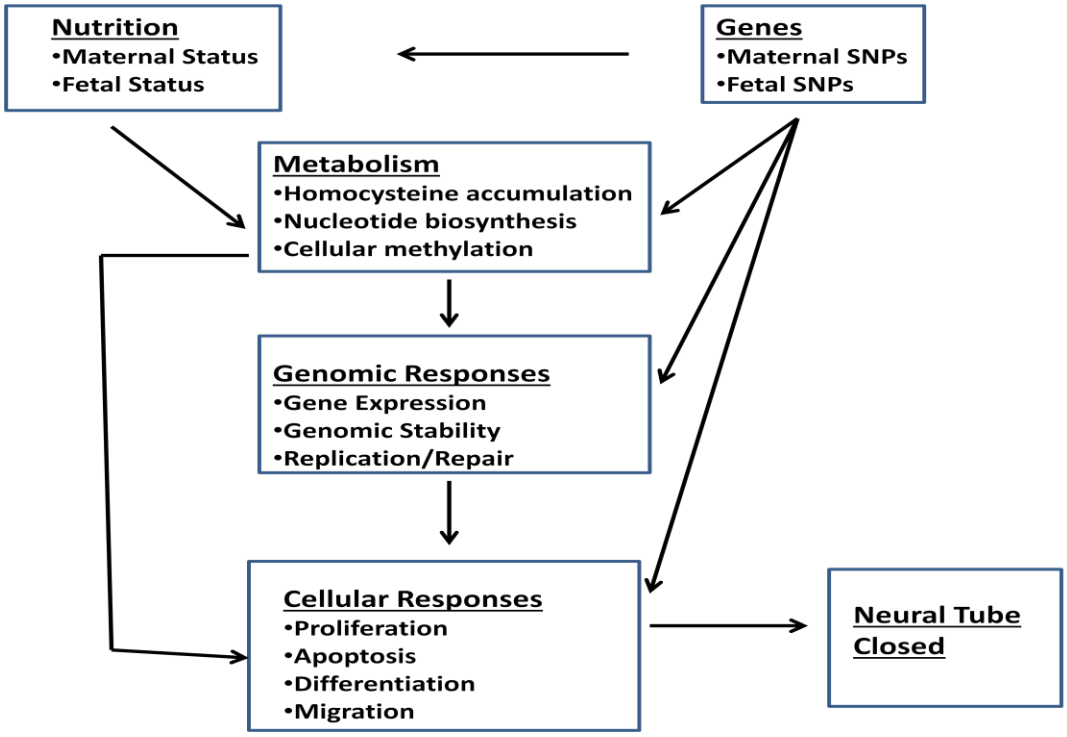

Figure 1.2 Folate status and NTDs. Image taken from Beaudin AE and Stover PJ. (2007). *Birth Defects Research.* **81:**183-203. The mechanism underlying folate status and NTDs remains unknown.

As previously stated there are a number of theories surrounding the underlying link between folate status and NTDs. One such theory is the "methylation hypothesis" which proposes that folic acid intervention prevents neural tube defects by stimulating cellular methylation reactions. [22] Homocysteine is a cytotoxic sulphur containing amino acid which can prevent methylation from occurring. Levels of homocysteine and folate are intrinsically balanced with an increase in folate causing a reduction in homocysteine. Evidence to support this theory includes the MTHFR 677C>T polymorphism. MTHFR makes one carbon units available for methylation reactions at the expense of purine and thymidine synthesis. The reduced activity of the TT phenotype causes a re-distribution of one-carbon units and this phenotype also displays a global reduction in DNA methylation which combined with low folate status may increase NTD risk. [22]

A second theory proposing a potential mechanism between folate status and NTDS is impairment in the *de novo* nucleotide synthesis pathway. Rapid growth during neural tube closure requires increased dependency on *de novo* nucleotide biosynthesis to sustain the rate of cell division. Any impairment in this pathway could lead to genomic instability as a result of uracil mis-incorporation which may affect neural tube closure.[23]

Clear evidence exists to indicate a genetic component to the developments of NTDs. Not only is there a difference in the number of NTDs between different ethnic groups, there is also evidence to suggest that that the risk of NTDs can be increased 3-8% in siblings of those affected with an NTD with evidence of increased risk in second and third degree relatives also. [24]

A review of the genetics of neural tube defects in humans by Greene *et al* lists 84 potential gene candidates associated with NTDs, 37 of which are involved in one-carbon metabolism. [18] SNPs in genes contributing to both the methylation cycle as well as the nucleotide synthesis pathway have been associated with NTDs.

The MTHFR 677 C>T polymorphism was one of the first, and still one of the most widely studied polymorphisms, associated with NTDs. The SNP causes an alanine to valine substitution which produces a thermolabile enzyme with a 50-60% reduction in homozygous individuals.[25] A meta analysis of the MTHFR 677C<T association confirmed an elevated risk of 50-70% for maternal TT genotype and 80-90% increase for fetal genotype. There also appears to be a slightly elevated risk in heterozygous individuals with mothers displaying a 10% increase and

offspring a 30% increased risk.[22] Other folate related gene polymorphisms such as the MTRR 66A>G polymorphism show similar results with some populations showing an association [26] while others show no association.[27] The varying of association of different SNPs between different populations is a common result. One theory to explain the lack of association between SNPs in genes, particularly those that regulate homocysteine remethylation, and NTDs is the lack of penetrance of these SNPs. This minimal effect of the SNPs suggests the possibility that genetic mutations which produce greater impairment are embryonic lethal.[21]

Another polymorphism strongly associated with maternal risk of NTDs is the MTHFD1 R653Q SNP. The mechanism underlying how the R653Q polymorphism works is believed to be by altering metabolic outcomes as no affect in folate or homocysteine levels have been reported therefore it may be the result of a negative effect on the *de novo* nucleotide synthesis pathway.[28]

The strong link between folate status and NTDs is well established. Folate status and folate gene polymorphisms have also been linked with other foetal malformations such as cleft lip and palate, limb deficiencies, birth defects of the heart, urinary tract defects and also increased risk for Down syndrome.[29] However as with NTDs the evidence would suggest that the underlying mechanism behind any and all of these birth defects is multi-factorial with environmental as well as genetic factors playing a role.

1.3.2 One-carbon metabolism and cancer

There is also an established link between one-carbon metabolism and the development of certain cancers. Folate deficiency can stress the mechanisms of DNA repair through increased uracil mis-incorporation resulting in DNA strand breaks and chromosome instability.[30] There is evidence to suggest that low folate status increases the risk of colon, breast, and pancreas cancer among others.[31] Alternatively high concentrations of folate have also been linked to carcinogenesis including prostate, lung and ovarian cancers.[32] The evidence would suggest that a delicate balancing act of folate status is required in order to prevent the risk of carcinogenesis.

However as with most diseases cancer has a tangled web of mechanisms underlying not only the development but also the sustainment of tumours. Therefore folate status alone is often not enough to create an increased risk of developing cancer, gene polymorphisms often have a role to play as well.

A study carried out by Suzuki et al [33] on a Japanese population found that low folate status combined with the MTHFR 677TT genotype increased the risk of breast cancer in post-menopausal women. The same study also found a correlation with low folate intake and the MTRR 66GG genotype and increased risk of breast cancer in post menopausal women. This study is just one example of the interactions between folate status and gene polymorphisms for one type of cancer. Similar studies have also shown a correlation between folate gene polymorphisms and folate status for a range of other cancer types including prostate [34] and colon [35] to name but two.

Combined with gene polymorphisms, folate status also affects DNA methylation patterns which in turn can affect gene expression patterns. Evidence to date suggests that the effects of folate deficiency on DNA methylation can depend on cell type, target organ and stage of transformation as well as being gene specific and/or site specific which can make it difficult to pinpoint the exact contribution folate status has on DNA methylation for different types of cancers.[36]

1.3.4 One-carbon metabolism and cardiovascular disease

Cardiovascular disease (CVD) is caused by disorders of the heart and blood vessels and includes heart attacks, stroke and high blood pressure. CVD is a widespread epidemic in Western populations with a number of factors contributing to its development. Impairment in folate mediated one-carbon metabolism, particularly in the methylation cycle, has been linked to CVD.

As already discussed homocysteine is a cytotoxic sulphur containing amino acid that is not sourced through the diet but is a product of the methylation cycle of folate metabolism. Levels of folate and homocysteine are linked with an increase in one leading to a decrease in the other. A high level of homocysteine, which indicates low levels of folate, is used as a marker for CVD.[37] There is still some debate as to whether raised homocysteine levels are a cause of CVD or just a useful indicator. A number of cohort and genetic polymorphism studies show an association between elevated serum homocysteine levels and increased risk of CVD, and there is some evidence to suggest that increased folic acid intake has a modest protective effect. There is however no conclusive evidence linking the two and opinion remains divided.[38]

As with other multi-factorial diseases polymorphisms in a number of folate related genes have been associated with CVD. Increased levels of homocysteine can be the consequence of a number of different gene deficiencies. These include inhibition of the trans-sulphuration pathway mediated by deficiencies in CBS. Alternatively increased homocysteine can be the result of the inhibition of the re-methylation pathway either directly by deficiencies in MS or indirectly by impairment to MTHFR.[39] The MTHFR 677 C>T polymorphism is again widely studied in relation to CVD. TT individuals display moderate hyperhomocysteinemia, the effects of which can often be stabilized with increased intake of folate.[39]

Polymorphisms in both TS and SHMT1, both of which are involved in the *de novo* thymidylate synthesis, have also been associated with increased risk of CVD in non-Hispanic white men. [40]

1.3.5 One-carbon metabolism and neurological disorders

Folate deficiency or homocysteine elevation has been shown to play a role in the development of a number of neurological disorders. In patients with Alzheimer's disease plasma homocysteine levels are generally increased, while low levels of folate, and also the enzyme SAM, are observed in spinal fluid.[41] A contributing factor to the development of Alzheimer's disease is the accumulation of amyloid β – peptide. There is some evidence to suggest that that increased concentration of homocysteine leads to increased concentrations of amyloid β – peptide leading to neurodegeneration. [42]

A second neurological disease linked to elevated homocysteine levels is Parkinson's disease. In a neuron cell culture model, used to study the disease, both folate deprivation and homocysteine made the cells more sensitive to damage and death, hastening the onset and progression of the disease.[43]

It was first observed in 1970 by Reynolds *et al* that there is a link between folate deficiency and depression.[44] It has since been shown that folic acid supplementation can reverse depression in some cases.[45] Although the exact mechanism underlying the link between folate deficiency and depression remains unclear it is hypothesized that it may involve hyperhomocysteinemia and altered methylation reactions.[46]

1.3.6 One-carbon metabolism and epigenetic regulation

Epigenetics is a mechanism for altering gene expression without altering the underlying genetic sequence. One method of epigenetic regulation is DNA methylation which alters the DNA by attaching a methyl group to cytosine residues particularly in CpG islands at the 5' end of the gene and thus changing gene expression patterns.[47] In one-carbon metabolism folate in the form of 5-methyltetrahydrofolate is involved in the re-methylation of homocysteine to methionine which is the pre-cursor to SAM which is the primary methyl donor for all cellular methylation reactions including DNA methylation.[48] A defining characteristic of epigenetic modification, unlike modifications to the DNA sequence, is that modifications to the epigenome are dynamic and can be strongly influenced by environmental factors such as folate status. A number of studies have been carried out on both human populations and mouse models to establish a link between folate status and DNA methylation. Most found that increased folate levels increased DNA methylation either globally or in a gene specific manner.[49,50] As previously discussed alterations of DNA methylation patterns can be associated with the development of certain cancers. Modifications in DNA methylation patterns are also important for embryonic development where DNA methylation plays a huge role in cell specific gene expression.[51] During pregnancy there is a positive correlation between folate status and DNA methylation. A study carried out by Kim *et al* [52] on a hyper-homocysteinemia rat model found that folate supplemented diet increased placental DNA methylation while a folate deficient diet decreased DNA methylation in placenta. Some research has also been carried out on the lasting effect of DNA methylation patterns on future health and disease however a defined relationship remains elusive.[53]

One-carbon metabolism is a complex network of biochemical reactions which provide two essential functions within the cell; 1) the building blocks of purine and thymidine synthesis essential for DNA replication and repair and 2) the most prominent methyl donor in the cell, SAM, which donates methyl groups for all cellular methylation reactions. Any impairment or deficiency within this pathway either environmentally, such as folate status, or genetically, including debilitating gene polymorphisms, can have devastating consequences and result in a broad spectrum of diseases including NTDs, CVD, cancer and Alzheimer's.

1.4 Dihydrofolate Reductase

Dihydrofolate reductase (DHFR) is the enzyme responsible for the reduction of dihydrofolate into tetrahydrofolate, a reaction which occurs in the presence of NADPH. DHFR forms part of the *de novo* thymidylate synthesis pathway along with thymidylate synthase (TS) and serinehydroxymethyltransferase (SHMT). DHFR is also the only known enzyme able to reduce folic acid, the synthetic form of folate. DHFR is a highly conserved enzyme that is found in all eukaryotes and prokaryotes. In protozoa it forms a gene fusion with another folate enzyme thymidylate synthase (TS).[54]

DHFR activity is tightly regulated at transcription, translation and at post – translation. Transcription of DHFR is regulated by a number of mechanisms including the binding of transcription factors and ncRNAs. The major DHFR promoter does not have a TATA or CAAT elements usually found in promoters but it does contain SP1 transcription factor binding sites and also E2F binding sites which allow regulation of DHFR transcription during the cell cycle with high expression levels during late G_1 and early S phase.[55] Transcription of DHFR is also regulated by an ncRNA which is produced by a minor upstream promoter. The ncRNA forms a stable complex within the major promoter which causes the pre-initiation complex to dissociate thus preventing transcription from occurring. [56]

DHFR has an auto-regulatory mechanism in place to control translation which involves the binding of DHFR protein to its own mRNA and suppressing translation.[57] This auto-regulatory mechanism can be disrupted by the chemotherapeutic drug methotrexate (MTX). The DHFR protein – mRNA complex undergoes a conformational change which results in the release of the mRNA and as a result increased levels of DHFR protein is often observed after initial treatment with MTX in mammalian cell culture models.[58]

On a post – translational level DHFR has been shown *in vitro* to undergo sumoylation by the protein SUMO-1 which is believed to occur *in vivo* during the S phase of the cell cycle to facilitate the translocation of DHFR to the nucleus where, along with thymidylate synthase and serine hydroxymethyltransferase (SHMT), it is part of the *de novo* thymidylate synthesis pathway.[59] Figure 1.3 summarizes the levels of regulation of DHFR activity.

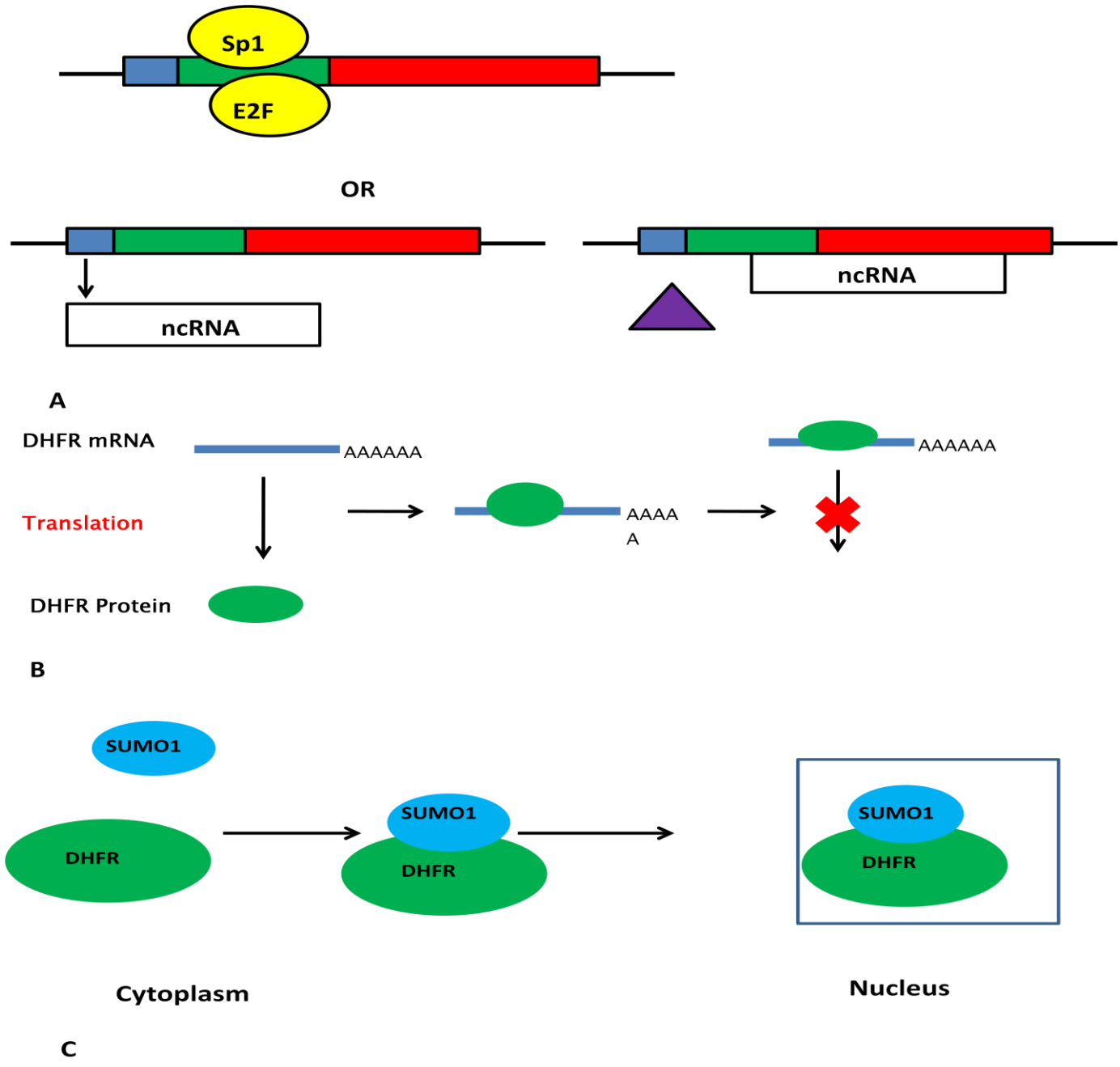

Figure 1.3 Regulation of DHFR activity. (A) DHFR activity is regulated at the transcriptional level by either the binding of transcription factors Sp1 or E2F or by an ncRNA coded for by the minor promoter. **(B)** Translation is auto-regulated by binding of the protein to mRNA to suppress translation. **(C)** Post-translational modification in the form of sumoylation is believed to support the transport of DHFR into the nucleus during S phase of the cell cycle where it is needed for *de novo* thymidylate synthesis.

The tight regulation of DHFR activity at all levels from gene expression through to the protein itself is due to the essential role it plays within the cell. Any deficiency in DHFR activity results in loss of tetrahydrofolate, the biologically active folate, and has a direct influence on DNA synthesis and cell proliferation.

This key role of DHFR in cell proliferation has made it the target of a number of antifolate drug therapies including the chemotherapeutic MTX which is used in the treatment of acute lymphoblast leukaemia (ALL) and also the autoimmune disease rheumatoid arthritis.[60] However the development of drug resistance is often a common problem. There are a variety of mechanisms by which resistance can occur. It can be acquired through amplification of the *DHFR* gene [61] or can occur through mutations to amino acids specific for MTX binding and affinity.[62] Other drugs such as trimethoprim and pyrimethamine target bacterial and protozoal DHFR with pyrimethamine being an effective treatment of malaria.[63]

Variations in the *DHFR* gene have also been linked to both disease susceptibility and response to treatment.[64] One of the most widely studied polymorphism of the *DHFR* gene is the 19bp insertion/deletion, which occurs in intron 1, particularly in relation to NTDs. Parle-McDermott et al reported that women with the deletion (del) allele had a lower risk of having a child with a NTD.[65] Gemmati D et al observed that the 19bp insertion/deletion polymorphism may have a protective role in patients with acute lymphoblastic leukaemia (ALL).[66] DHFR polymorphisms located in the 3' UTR region have been shown to impact on MTX efficiency. Three separate polymorphisms in this region 829C>T[67], 35289A>T[68] and1171A>T[69] have been reported to negatively affect response to methotrexate treatment

1.5 The Dihydrofolate Reductase Gene Family

Due to its role in health and disease DHFR has been extensively studied and continues to be of interest within the field. The functional human *DHFR* gene was first characterized by Chen et al in 1983 [70] and mapped onto chromosome 5. The DHFR gene family however also contains four processed pseudogenes, *DHFRP1*[71,72] *DHFRP2,* [71, 72] *DHFRP3*[73] *and DHFRP4/DHFRL1*, [71, 72] scattered across the genome. Pseudogene chromosome locations are listed in Table 1.1.

A pseudogene is characterized by its similarity to one or more paralogous gene but is non-functional[74] i.e. it is either not transcribed or translated into a functional protein.

The *DHFRP1* pseudogene which is located on chromosome 18 is polymorphic in the human population [72] and the ORF for this pseudogene is identical to that of the functional *DHFR* gene however it does not appear to have any promoter activity. *DHFRP2* contains several stop codons within the ORF making expression unlikely and the *DHFRP3* pseudogene only contains the 3' half of the DHFR coding sequence. [73] The fourth annotated pseudogene of DHFR, *DHFRP4* or as it is more commonly known *DHFRL1* (dihydrofolate reductase like 1) is located on chromosome 3 and has a 92% sequence homology to the functional DHFR. What distinguishes this pseudogene from the others is not only an ORF without in-frame stop codons and promoter activity but also the necessary signals for translation. The translation signal for DHFRL1 differs from that of DHFR by one base; ctgtcAUGt (DHFRL1) *versus* ctgtcAUGg (DHFR). A recent study reported that up to 50% of cytoplasmic translated transcripts do not contain a g at the + 4 position[75] so it is unlikely that the base difference would prevent translation from occurring.

A large scale cDNA sequencing project conducted in 2004[76] indicated that the *DHFRL1* gene is actually expressed. Annotation of the *DHFRL1* entry in the Ensembl database (www.ensembl.org) suggests that the DHFRL1 gene actually produces two transcripts, T1 and T2, which differ in their 5'UTR region but produce the same protein sequence. An RT-qPCR experiment previously conducted in the lab by Dr. Kirsty O'Brien confirmed that the T2 transcript is expressed in a number of human cell lines.

Table 1.1 *DHFR* gene family and their chromosome locations

Gene Name	Type	Chromosome Location
DHFR	Functional	5 *
DHFRP1	Processed Pseudogene	18 *
DHFRP2	Processed Pseudogene	6 *
DHFRP3	Processed Pseudogene	2 **
DHFRP4 **or** ***DHFRL1***	Processed Pseudogene or Retrogene?	3 ***

*Anagnou *et al*., (1984, 1988)

** Shimada *et al*., (1984)

*** Maurer *et al*., (1985); Anagnou *et al*., (1984, 1988)

1.6 Pseudogenes and their role within the cell

The publication of the human genome in 2001 was a major milestone in the study of human genetics and revolutionized the field. One of the major revelations was that only 1% of the human genome consists of protein coding genes, the other 99% consists of non-coding sequences which includes but is not limited to pseudogenes. The classic definition of a pseudogene is a gene copy that has acquired mutations such as frame shifts or premature stop codons which have made them non-functional.[77] The first pseudogene was reported in 1977 by Jacq *et al* in *Xenopus laevis* for oocyte-type 5 RNA [78] however many more have been reported since then in a variety of species including plants, insects and even bacteria. [79] There are two categories of pseudogene; the first and most common is a processed pseudogene which is formed through the re-integration of an mRNA intermediate. The second type is a non-processed pseudogene which most likely arose due to a gene duplication event and contains both exons and introns (Figure 1.4). Not all non-processed pseudogenes are the result of gene duplication some evolve when a single copy parent gene becomes non-functional. These types of non-processed pseudogene have been named a unitary pseudogene [80] and have been used to study functional losses over time.

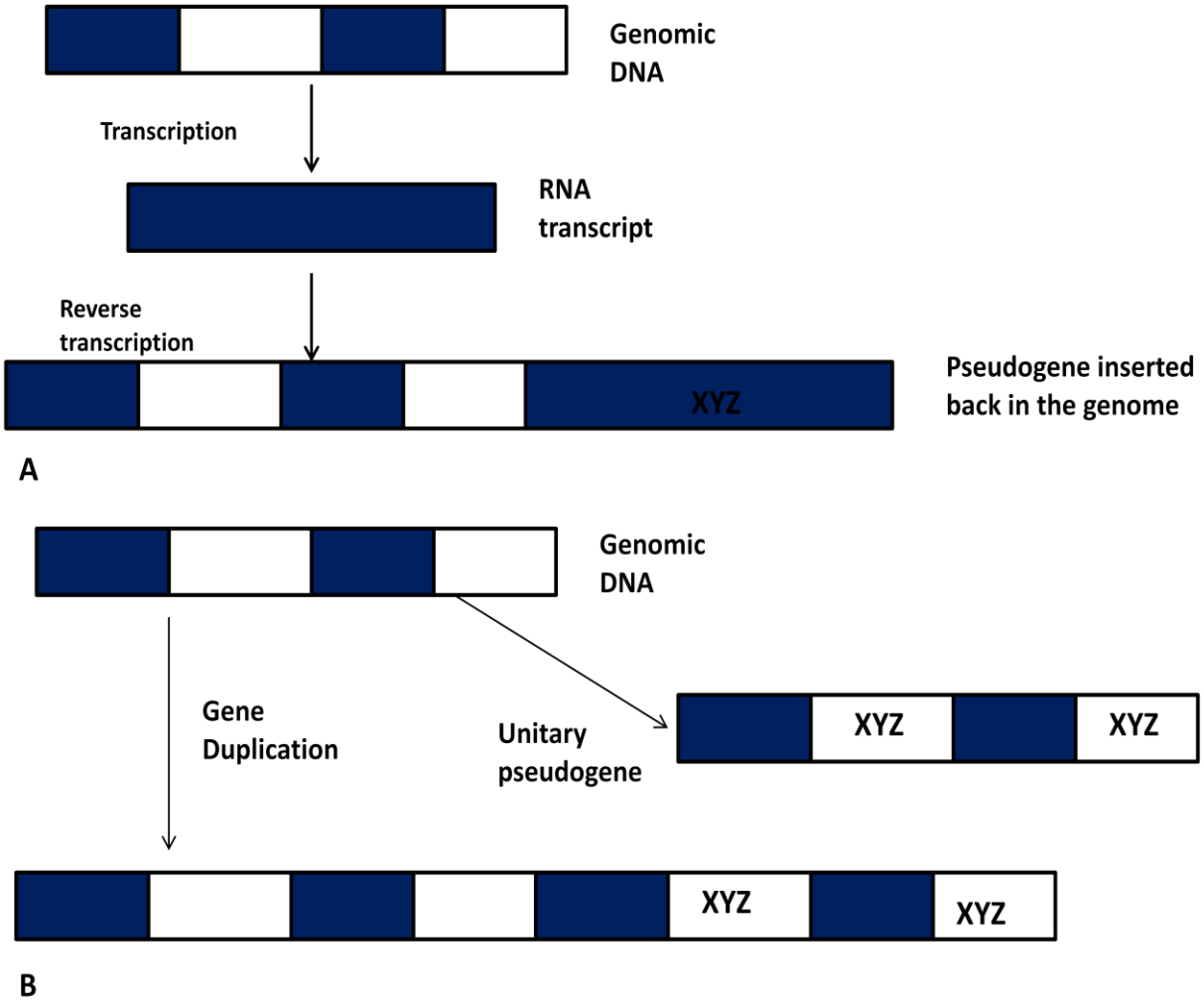

Figure 1.4 Formation of pseudogenes. (A) Processed pseudogenes, the most common form, arose through the re-integration of an mRNA intermediate which usually acquired a debilating mutation. (XYZ denotes mutations). **(B)** Non-processed pseudogenes arose either through a gene duplication event with the duplicated gene usually acquiring detrimental mutations or when a single copy parent gene acquires mutations which make it non-functional. These are known as unitary pseudogenes and are useful in loss of function studies.

Is has been estimated that there are roughly 20,000 pseudogenes in the human genome, [81] the question arises why pay a costly energy bill to maintain pseudogenes if they are non-functional? The ENCyclopedia of DNA Elements (ENCODE) project, which studies a representative 1% of the sequence of the human genome, found that at least 20% of pseudogenes are actively transcribed. Interestingly this project also revealed that approximately 80% of human processed pseudogenes are primate specific indicating a high level of retro transposition in primates.[82] This high level of transcription would indicate that pseudogenes are not just dead genomic "junk" DNA but has a functional role within the cell.

A number of pseudogene transcripts have been found to act as mRNA regulators through a variety of mechanisms. It has been proposed that pseudogenes antisense transcripts can combine with sense genic transcripts to control levels of expression. A study by Hawkins *et al* found that knockdown of an RNA antisense to an *Oct4* pseudogene led to an increase in expression of both the parent *Oct4* gene two of its other pseudogenes.[83] Two separate studies carried out by Tam *et al* [84] and Watanabe *et al* [85] found that in mouse oocytes some pseudogene transcripts can become small interfering RNAs (siRNAs) and silence gene expression. These siRNAs were formed by one of two ways; an internal secondary hairpin structure in the pseudogene transcript or from double stranded RNA composed of sense and antisense transcripts, either pseudogene-pseudogene or pseudogene-coding mRNA. A third mechanism of regulation may be the competition of pseudogene transcripts with coding transcripts for trans-acting elements such as microRNAs (miRNAs). Poliseno *et al* [86] found that a number of miRNAs which target *PTEN*, a tumor suppressor protein, also target the pseudogene *PTENP1* with the pseudogene transcripts acting as decoys by binding to the miRNA and allowing the PTEN transcripts to be translated. Figure 1.5 taken from Pink *et al* [87] summarizes the possible mechanisms by which pseudogene transcripts can act as regulators within the cell.

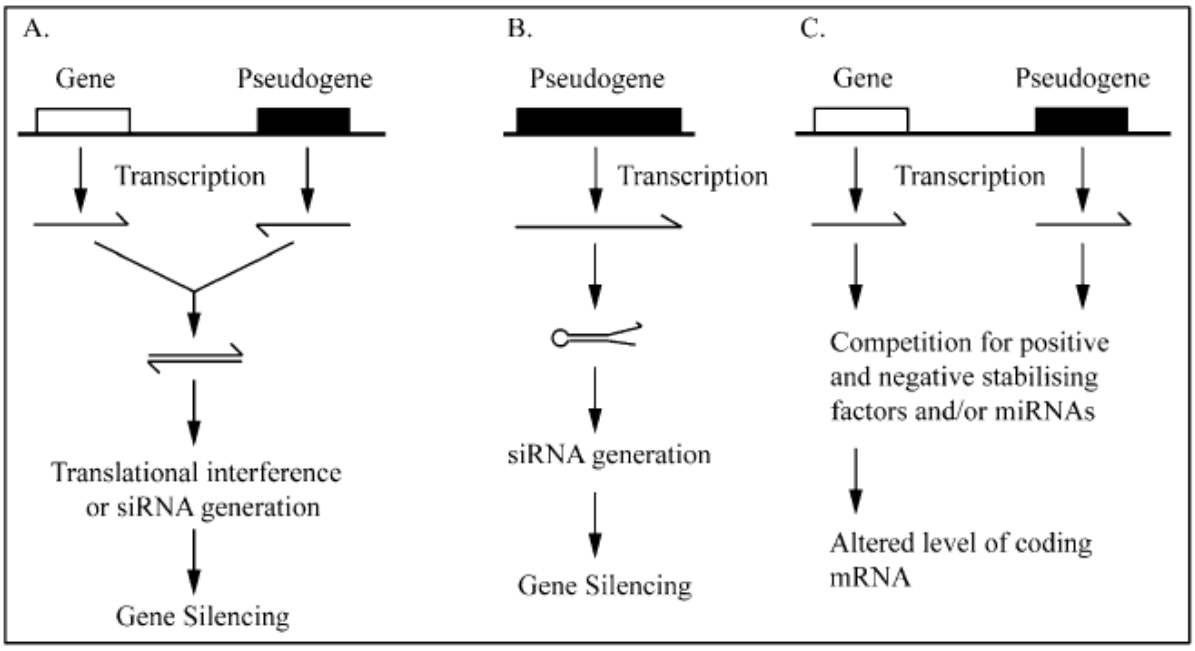

Figure 1.5 Potential mechanisms of pseudogene function. Image taken from Pink *et al* (2011). *RNA*. **17(5):**1-8. There are three proposed mechanism of pseudogene RNA function. **(A)** anti-sense pseudogene RNA can combine with sense strand RNA from another homologous coding gene to either produce siRNA or interfere with translation. **(B)** Secondary hairpin structures in the pseudogene transcript can form siRNA molecules resulting in gene silencing. **(C)** Pseudogene transcripts can compete with their coding counter parts for trans acting elements such as miRNA which in turn will alter the expression levels of the coding mRNA.

Pseudogenes may not only function as RNA regulators, they may also act as DNA storage depots that can give rise to new genes. The human *XIST* ncRNA, which is involved in X chromosome inactivation, is known to have arisen from a pseudogene. It is also well known that pseudogenes can increase antibody diversity through gene conversion events with their parent gene.[88]

There are few examples of translated pseudogenes; however there are a few reported cases. In 2000 Moreau-Aubry *et al* [89] reported a translated pseudogene in melanoma cells. The pseudogene in question was a processed pseudogene of CTL, which recognizes antigens presented by tumor cells. The pseudogene itself codes for a new antigen which causes a CTL response against the melanoma by activating T cells. [86]

In 2004 Pai *et al* discovered that a pseudogene of cytochrome P450 called *CYP2D7* was translated into a functional protein that leads to the demethylation of codeine into morphine in the brain. [90] The same study also revealed that this pseudogene is only translated in the brain and not the liver or kidneys, which are also major centres for drug metabolism. The pseudogene itself contains a deletion at position 138 which enables it to metabolise codeine into morphine at a higher rate.[87]

If, as the evidence would suggest, a number of pseudogenes are transcribed into mRNA why are there so few reported cases of pseudogene transcripts translated into proteins? It may be possible that few pseudogenes are actually translated into proteins and their role within the cell is purely at the DNA and RNA level to contribute to gene evolution and act as gene expression regulators. It may also be possible that any potential pseudogene proteins may not be detected after translation particularly if they are highly similar to the established parent protein, it may be difficult to distinguish between the parent protein and the pseudogene protein at a molecular level. Another consideration is that to date any experimental evidence proposed for the functionality of pseudogenes have been focused on specific gene families, some of which may only function at the RNA level. Considering the prevalence of pseudogenes in mammalian genomes and the increasing realization that they have a role to play within the cell it is possible that more functional pseudogenes, perhaps at both an RNA and protein level, are waiting to be discovered which may give us a better understanding of the transcriptome and possibly the proteome. Pseudogenes that produce a functional product, either RNA or protein, are not true pseudogene and all such cases need to be re-annotated correctly

1.7 Aims/Objectives

To date it has been assumed that DHFR is the only enzyme capable of reducing dihydrofolate into tetrahydrofolate in humans. Confirmation of the expression of a *DHFRL1* transcript, a transcript from an annotated pseudogene, opens up the possibility that if this transcript is translated then there is potentially a second dihydrofolate reductase enzyme in humans. Preliminary bioinformatic analysis predicts that the DHFRL1 protein could function as a dihydrofolate reductase enzyme but with reduced catalytic efficiency, it may be an inefficient binder of MTX, it could possess the capability of regulating the translation of both itself and DHFR and it may undergo sumoylation.

The aim of this project is to investigate whether *DHFRL1* is an actual pseudogene as previously thought, or is it in fact a functional retrogene. This will include:

1. **Assessment of dihydrofolate reductase activity in DHFRL1**

The primary function of DHFR is to reduce dihydrofolate to tetrahydrofolate. In order to determine if *DHFRL1* is a pseudogene or functional retrogene it is inportant to establish if DHFRL1 protein has enzyme activity and how this compares to DHFR enzyme activity. Initial bioinformatic assessment of DHFRL1 amino acid sequence reveals the presence of catayltic domains, however a substitution at position 24, W24R, may effect substrate binding reducing any potential catalytic activity.

2. **Investigating the role of DHFRL1 in regulating both itself and DHFR**

DHFR has the ability to regulate its own translation by binding to its own mRNA in order to suppress translation. Once again bioinformatic analysis reveals that the amino acids required for RNA binding are present in DHFRL1 protein. Due to the similarity between DHFR and DHFRL1 at both RNA and protein level the potential binding of DHFRL1 protein to DHFR mRNA, as well as DHFR protein binding to DHFRL1 mRNA also needs to be established.

3. DHFRL1 as a therapeutic target

DHFR is the target of a number of anti-folate drug therapies including the chemotheraputic methotrexate (MTX). timepoints. The potential presence of a second dihyrofolate reductase enzyme and its effect on therapeutic strategies needs to be established. Determining the binding affinity, Ki, of DHFRL1 to MTX will be an important first step. Also as MTX disrupts the DHFR auto-regulation mechanism examining its effect on DHFRL1 in this way also needs to be investigated.

4. DHFRL1 expression and protein localisation in the cell

Folate metabolism occurs in the cytoplasm, mitochondria and nucleus of the cell. DHFR is present primarily in the the cytoplasm. During the S phase of the cell cycle, where DNA synthesis is at its peak, *DHFR* expression is increased and the protein undergoes sumoylation to the nucleus.

Determining the sub cellular localisation of DHFRL1 protein is important for elucidating the role of DHFRL1 in the cell. As DHFRL1 protein also contains a sumoylation motif, investigating the potential of DHFRL1 to be sumoylated and also determining expression levels of *DHFRL1* during the phases of the cell cycle may determine if DHFRL1 is also present in multiple organelles.

5. Investigating possible functional *DHFR* retrogenes in other mammals

If the presence of a second dihydrofolate reductase enzyme in humans can be confirmed, it may be possible that a second enzyme exists in other mammals also. It will be important to establish the presence or absence of a second enzyme in other mammals as they are frequently used as model organisms. Mice in particular are a common model organism used to study folate metabolism

CHAPTER 2

Materials and Methods

2.1 Materials

2.1.1 Reagents

Mammalian Cell Culture

Gibco Iscove's Modified Dulbecco's Medium (IMDM) 1X (Cat No. 21980), Dulbecco's Modified Eagle Medium (DMEM) 1X (Cat No. 41965), Dulbecco's Phosphate Buffered Saline (DPBS) 10X (Cat No. 14200), Trypan Blue Stain 0.4% (Cat No. 15250-061).

Sigma Aldrich Sodium Pyruvate (Cat No. S8636), Trypsin-EDTA (Cat No. T4049), Penicillin-Streptomycin (Cat No. P4333), G418 (Cat No. A1720), Nocodazole (Cat No. M1404), L-mimosine (Cat No. M0253), Thymidine (Cat No. T1895), Rnase A, (Cat No. R4642), Propidium iodide (Cat No. P4864).

Cruinn Tissue Culture flasks 75cm^2 (Cat No. 658175CI), Tissue Culture flasks 25cm^2 (Cat No. 690175CI), Cell Scrappers (Cat No. 541070G)

Biosera Fetal Bovine Serum (FBS) (Cat No S1900)

Invitrogen Lipofectamine 2000 reagent (Cat No 11668500), MitoTraker CMTMROS (Cat No M7512)

Roche Cell Proliferation Assay (MTT) (Cat No 11465007001)

Bacterial Cell Culture

Sigma Aldrich Tryptone (Cat No. T7293), Agar (Cat No. A5306), Yeast Extract (Cat No. Y1333), NaCl (Cat No. S7653), Ampicillin (Cat No. A0166), Kanmycin (Cat No. K4000), Streptomycin (Cat No. S6501) Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Cat No. 15502)

Molecular Biology

Sigma Aldrich Agarose (Cat No. A9539), Orange G (Cat No. O3756), RNA Loading Buffer (Cat No. R1386), Boric Acid (Cat No. B6768), Tris (Cat No. T6066), Ethylenediaminetetraacetic acid (EDTA) (Cat No. E9884), Ethanol (Cat No. E7023),

New England Biolabs (NEB) 1kB Ladder (Cat No. N04685), 100bp Ladder (Cat No. N3231)

Bioline ISOLATE Plasmid Mini Kit (Cat No. BIO-52026), ISOLATE RNA Mini Kit (Cat No. BIO-52043), BioScript (Cat No. BIO-27036), Oligo dT (Cat No. BIO-38029), Random Hexamers (Cat No. BIO-38028), SensiFast™ Probe One Step Kit (Cat No. BIO-76001) MyTaq (Cat No. BIO21111).

Roche Probe #24 (Cat No. 04686985001), Probe #57 (Cat No. 04688546001), Probe # 89 (Cat No. 04689143001), Probe # 64 (Cat No 04688635001), Probe # 16 (Cat No. 04686896001), Probe # 9 (Cat No. 04685091001), Probe # 17 (Cat No. 0468690001).

Agilent Technologies QuikChange II XL lit (Cat No. 200522).

Protein Expression and Analysis

Sigma Aldrich DHFR Assay Kit (Cat No. CS0340-1KT), Gluthione (Cat No. G6013), Sodium dodeyl sulfate (SDS) (Cat No. L4390), Ammonium Persulphate (APS) (Cat No. A3678), Acrylamide/Bis (Cat No. A9926), β-mercaptoethanol (Cat No. M6250), Glycine (Cat No. G8898), Coomaissie Blue (Cat No. B8647), Bovine Serum Albumin (Cat No. A2153), Trition X100, (Cat No. X100), Tween (Cat no. P1379), GAPDH antibody (Cat No. G8795), Anti-Goat HRP secondary antibody (Cat No. A5420), Anti- Mouse HRP secondary antibody (Cat No. A3682), Anti-Rabbit HRP secondary antibody (Cat No. A0545), Acetone (179124), Freund's Complete Adjuvant (Cat No. F5881), Freund's Incomplete Adjuvant (Cat No. F5506).

Fisher Scientific Gluthione Agarose (Cat No. PN16100), SuperSignal Femto (Cat No. PN34094), protease inhibitors (Cat No. BPE9706), Methanol (BPE1105), Acetic Acid (Cat No. A/0400/PB17), TMB substrate (Cat No. PNN301), 96 well microplates (Cat No. FB56426), EMSA kit (Cat No. PN20148X), RNA Biotinylation Kit (Cat No PN20160), BCA reagents (Cat No. PN23228).

Bioline HyperPAGE Protein Marker (Cat No. BIO33065)

Medical Supply Company Rabbit Reticulocyte Lysate (Cat No. L4960).

Active Motif Sumolink™ SUMO-1 kit (Cat No. 40120)

Abcam DHFR antibody (Cat No. ab85056), EGFP antibody (Cat No. ab32146).

Invitrogen TEV protease (Cat No. 12575015), iBlot transfer stacks (Cat No. IB3010).

Cambridge Bio-Sciences PDH antibody (Cat No. MSP03)

Qiagen Mitochondria Isolation Kit (Cat No. 37612).

2.1.1b Instruments

MiniBis Pro Bio-Imaging Systems

GeneGnome Syngene Bio-Imaging

Innova®43 Incubator Shaker Series New Brunswick Scientific

GeneAmp® PCR System 9700

Roche Lightcycler® 480

Invitrogen iBlot

Bioair Safeflow 1.2

Thermo Scientific Forma Steri-Cycle Co_2 Incubator

Olympus CKA31 Microscope

Tecan i600 spectohotometer

Thermo Scientific Nanodrop

2.1.2 Stock Solutions

LB Broth – 2.5g Tryptone, 2.5g NaCl, 1.25g Yeast Extract, 250ml Deionised H_2O

LB Agar – 15g of agar per liter of LB Broth

Orange G (10X) – 0.1g Orange G, 20g Sucrose, 50ml H_2O

TBE (10X) – 48.44gTris HCl, 12.37g Boric Acid, 1.5g EDTA, 500ml H_2O

PBS (10X) – 80g NaCl, 2g KCl, 14.4g Na_2HPO_4, 2.4g KH_2PO_4, 1L H_2O (pH7.4)

TBS (10X) – 24.23g Tris HCl, 80.06g NaCl, 1L H_2O (pH 7.6)

TBS-T – TBS + 1% Tween

Tris – Glycine (5X) – 15.1g Tris Base, 94g Glycine, 50ml 10% SDS, 950ml H_2O

Transfer buffer (10X) – 15.14g Tris HCl, 72.07g Glycine, 500ml H_2O

Coomassie Blue Stain – 2.5g Coomassie, 100ml Acetic Acid, 300ml Methanol, 600ml H_2O

Coomassie Blue De-stain – 10ml Acetic Acid, 30ml Methanol, 60ml H_2O

Protein Lysis Buffer –50mM Tris, pH 7.8, 1mM EDTA, 0.1% v/v Triton X-100, 10mM β-mercapthoethanol, protease inhibitors (500μM 4-(2-Aminoethyl) Benzenesulfonyl Fluoride Hydrochloride, 150nM Aprotinin, 1μM E-64, 1μM Leupeptin, 0.5mM EDTA)

2.1.3 Cell Lines

CHO DG44 (Chinese Hamster Ovary)	DSMZ (Cat No. ACC 126)
HEK 293 (Human Embryonic Kidney)	ATCC (Cat No. CRL-1573)
J774 (Mouse Macrophage)	ATCC (Cat No. TIB – 67)
NRK 52E (Rat Kidney Epithelial)	DSMZ (Cat No. ACC 199)
Escherichia coli D3-157	ATCC (Cat No. 47050)

2.2 Cell Culture Methods

2.2.1 Mammalian Cell Culture

The cell line CHO DG44 were cultured in IMDM medium supplemented with 10% (v/v) fetal bovine serum (heat-inactivated), 1% (v/v) Penicillin Streptomycin, 0.1mM Hypoxthine and 0.016mM Thymidine. HEK 293, J774 and NRK cell lines were cultured in DMEM medium supplemented with 10% (v/v) fetal bovine serum (heat-inactivated), 1% (v/v) Penicillin Streptomycin, 200mM L-Glutamine. All cell lines were maintained at 37^{o}C in the presence of 5% CO_2 in their respective growth media. Cells were passaged by incubating with 0.25% Trypsin-EDTA at 37^{o}C for 5-10 minutes. The trypsin was in-activated by addition of growth media and cells were collected by centrifugation at 500 x g for 5 minutes. The sub-cultivation ratio was 1:3 – 1:5.

2.2.2a Cell Viability and Cell Counts

After collection of cells by centrifugation the cell pellet was resuspended in 1ml of complete media. The cell suspension was then added to trypan blue in a 1:10 dilution and left at room temperature for 5 minutes. The cells were then applied to a haemocytometer and viewed under a microscope at 100x magnification. Dead cells appear blue under the microscope and live cells are white as they can exclude the dye. The number of live cells were counted in the four corners (each corner has 16 squares). The average cell count was calculated from all four corners and multiplied by the dilution factor and then by 10^4 as cells were resuspended in 1ml.

2.2.2b MTT Assay

MTT assays were performed using a Cell Proliferation Kit I [MTT] (Roche). Cells were trypsinized as normal and a cell count performed using a haemocytometer. A standard curve of known concentrations of cells was seeded in a 96 well plate. All cells were seeded in triplicate. The cells were then incubated overnight at 37^{o}C, 5% CO_2. After incubation 10µl of MTT labeling reagent (final concentration of 0.5mg/ml) was added to each well. The plate was then incubated at 37^{o}C, 5% CO_2 for 4 hours. After incubation 100µl of solubilization solution was

added to each well and the plate was incubated overnight at 37^{o}C, 5% CO_2. Absorbance was then read at 550nm using a Tecan iControl spectrophotometer.

2.2.3 Transfection

Cells were seeded in a 6 well plate at a density of 2 x 10^5 cells/ml incomplete growth media and incubated at 37^{o}C for 24 hours. The media was removed from the cells and replaced with respective growth media containing no FBS. A total of 5µg of plasmid DNA was transfected into the cells using Lipofectamine 2000 reagent. Six hours after transfection the media was replaced with complete growth media. For stable transfections 500µg/ml of G418 was added to cells 48hours after transfection.

2.2.4 Sub-cellular localization by immunofluorescence

All cell types (HEK 293, J774 and NRK), grown on cover slips (1x10^5cells/ml), were transfected with 5µg of GFP tagged plasmid DNA using Lipofectamine 2000 reagent. Six hours after transfection, the transfection medium was removed and replaced with complete growth medium. The cells were re-transfected again 24 hours after the initial transfection using the same conditions. A further 48 hours later, the cells were incubated for 20 minutes at 37^{o}C with MitoTraker CMTMROS at 200nM. The cells were then fixed in paraformaldehyde on ice for 30 minutes. Following rinsing 3 x 5 min in PBS (Phosphate Buffered Saline) baths, the cover slips were mounted on slides with antifade medium (Dako). Slide preparations were observed using a Zeiss Axio Observer. Z1 equipped with a Zeiss 710 and ConfoCor3 laser scanning confocal head (Carl Zeiss, Germany). Images were analyzed using Zen 2008 software.

2.2.5 Cell cycle arrest

Method essentially as described by Jackman et al.[91] For cell cycle arrest experiments HEK 293 cells were cultures as described in section 2.2.1. For each experiment cells were seeded at $5x10^5$ cells/ml in a T75 flask.

Enrichment G_2/M phase by Nocodazole

Cells were seeded in complete growth media and incubated at 37°C, 5% CO_2 for 24 hours. Nocodazole was added to the cultures at a final concentration of 400ng/ml and cells were incubated for a further 12 hours. Cells were then washed in complete growth media and 12ml of fresh complete growth media was added to the culture and incubated for 4 hours at 37°C. Cells were trypsinized and a cell count performed; $2x10^5$ cells were used for FACs analysis and the rest were used for RNA isolation.

Enrichment of G_0/G_1 phase by serum starvation

Cells were seeded in DMEM media containing 0.5% fetal bovine serum and incubated at 37°C for 24 hours. Cells were trypsinized and a cell count performed; $2x10^5$ cells were used for FACs analysis and the rest were used for RNA isolation.

Enrichment of G_1/S phase by L-mimosine

Cells were seeded in complete growth media which also contained 400µM L-mimosine. Cells were incubated at 37°C for 24 hours. The media was removed and replaced with complete growth media and cultures were incubated for a further 24 hours. Cells were trypsinized and a cell count performed; $2x10^5$ cells were used for FACs analysis and the rest were used for RNA isolation.

Enrichment of S phase by double thymidine block

Cells were seeded in complete growth media which also contained 2mM thymidine. Cells were incubated at 37^0C for 12 hours. The media was removed and cells were washed twice with complete growth media. Cells were incubated for 16 hours in complete growth media. The media was removed and replaced with media containing 2mM thymidine and cells were incubated at

37°C for a further 12 hours. Cells were trypsinized and a cell count performed; $2x10^5$ cells were used for FACs analysis and the rest were used for RNA isolation.

Preparation of samples for FACs analysis

After cell count was performed cells were washed in ice-cold PBS. Cells were resuspended in 200µl PBS and vortexed for 10 seconds. 4ml of ice-cold 70% ethanol was added to the cells and incubated on ice for a minimum of 45 minutes (samples can be stored at -20°C overnight at this point). Samples were centrifuged at 200x g for 10 minutes at 4°C. The supernatant was removed and the pellet was resuspended in 1ml of PI master mix (propidium iodide 40µg/ml, RNase 100µg/ml, PBS in a total volume of 1ml). Samples were wrapped in tinfoil to protect from the light and then incubated at 37°C for 30 minutes before analysis by flow cytometry.

2.2.6 Bacterial Cell Culture

Escherichia coli D3-157 were cultured in LB Broth containing 0.05mg/ml thymidine and 0.1mg/ml streptomycin. DH5α and BL-21A1 cells were cultured in LB Broth containing 50ug/ml Ampicillin. All cultures were grown at 37°C at 220rpm. Glycerol stocks of bacterial cultures were made by adding 700µl of overnight culture to 300µl of 50% glycerol stock solution and stored at -80°C.

2.2.7 Transformation of bacterial cells

An aliquot of 50µl of cells was thawed on ice. Cells were mixed gently and 10ng of DNA in a total of 5µl was added to each aliquot. Cells were incubated on ice for 1 hour. Cells were then heat shocked at 42°C for 2 minutes. SOC medium or LB medium without antibiotics was added to the cells in a total volume of 250µl and cells were incubated at 37°C, 200rpm for 1 hour. A volume of 20µl and 100µl of transformed cells were spread on pre-warmed selective LB agar plates and incubated overnight at 37°C.

2.3 Molecular Biology Methods

2.3.1 Electrophoresis - Agarose gels

Agarose gels were made by boiling 1% agarose (w/v) in 1xTBE. Ethidium bromide (10µg/ml) was added to liquid agarose and cooled to 55^{o}C before pouring into the gel mould. The electrophoresis buffer was also 1xTBE. Samples were mixed with orange G loading dye and electrophoresised at 90 volts for approx 45 minutes and were visualized under UV light using DNR Mini-Bis Pro Bio-Imaging System.

2.3.2 Plasmid DNA Isolation

Overnight bacterial cultures were centrifuged at 1500 x g for 10 minutes to pellet cells. The supernatant was removed and the pellet was resuspended in re-suspension buffer from ISOLATE Plasmid Mini Kit (Bioline). The plasmid DNA was then isolated as per manufacturer's instructions. Plasmid DNA was then visualized on a 1% agarose gel.

2.3.3 RNA Isolation

Cells were lysed at 80% confluency directly using the lysis buffer from ISOLATE RNA Mini Kit (Bioline) and the RNA was isolated as per manufacturer's instructions. RNA was visualized on a 1% agarose gel by adding 1µg of RNA to RNA denaturing buffer (Sigma) and heating to 65^{o}C for 5min before loading onto gel. Good quality RNA should have 28S band approx twice the size of the 18S band. The 5S band may also be visible in some cases.

2.3.4 Estimation of nucleic acid concentration

Concentrations of both DNA and RNA were determined using a nanodrop (Thermo Scientific) measuring at A_{260nm}. Nucleic acids free from contamination with either salts or organic material should have A260:280 ratio of between 1.8 and 2.[92]

2.3.5 Reverse Transcription PCR (RT PCR)

RNA was isolated as described in 2.3.3 and any genomic DNA contamination was removed by treatment with 1µl DNase I at room temperature for 15 minutes, reaction was stopped by

incubating at 70°C for 10 minutes in the presence of 25mM EDTA. Reverse transcription using 2µg of RNA was carried out using a mixture of oligo dT (0.5µg/µl) and random hexamers (0.5µg/µl) in combination with Bioscript enzyme as per manufacturer's instructions. The reaction was placed on a thermocycler under the following conditions:

25°C	10 min
42°C	50 min
70°C	15 min
4°C	∞

2.3.6 Reverse Transcription Quantitive PCR (RT-qPCR)

RNA (2µg) was reverse transcribed as described above. All assays, with the exception of the rat DHFRLs which was manually designed, were designed using the Universal Probe Library (Roche, UK) and where possible were intron spanning. For full list of primers and probes used see Table 2.1. All assays and analysis were carried out using Sensimix™ II Probe Kit (Bioline) on the Roche Lightcycler® 480 instrument under the following conditions:

95°C	10 min	1 cycle
95°C	10 sec	
60°C	30 sec	45 cycles
72°C	1 sec	
72°C	10sec	1 cycle

For each RT-qPCR experiment a panel of endogenous controls was examined before one was selected to use in the experiment. A gene was only selected as the endogenous control if it showed the same level of expression across all experimental samples i.e. if the expression of the gene was unaffected by the conditions of the experiment. This was done to ensure that any changes observed in expression of test genes were not due to a change in the expression of the endogenous control.

2.3.7 Human DHFR/DHFRL1 Cloning

2.3.7a Gateway Cloning

GST expression clones

An entry clone for both DHFR and DHFRL1 was created by cloning PCR product amplified from HEK 293 cDNA, primers used in this reaction are shown in Table 2.1, into PDONR™ 211 vector, (Appendix 2.1 for vector map). An expression clone was then created by performing an LR reaction between plasmid DNA from the entry clone and pDEST™ 15 vector, (Appendix 2.2 for vector map). The recombination reaction was then transformed into Library Efficiency® DH5α cell (Invitrogen). Positive clones were selected for on LB Agar plates containing 50µg/ml ampicillin. Plasmid DNA was isolated, as described in section 2.3.2, and clones confirmed by both restriction digest and Sanger sequencing.

Mammalian expression clones

An entry clone for both DHFR (IOH4402) and DHFRL1 (IOH26763) was purchased from Invitrogen. An expression clone was then created by performing an LR reaction between plasmid DNA from the entry clone and pcDNA3.2/V5, (Appendix 2.3 for vector map), and pcDNA6.2, (Appendix 6.2 for vector map), vectors respectively. The recombination reaction was then transformed into Library Efficiency® DH5α cell (Invitrogen). Positive clones were selected for on LB Agar plates containing 50µg/ml ampicillin. Plasmid DNA was isolated, as described in section 2.3.2, and clones confirmed by both restriction digest and Sanger sequencing.

2.3.7b TA Cloning

pCR2.1 expression clones (*E-coli* complementation study)

The TA cloning system (Invitrogen) was used to construct a pCR2.1 expression vector with either DHFRL1 or a DHFR insert as per manufacturer's instructions. The template for each PCR was plasmid DNA from pDEST15-rDHFRL1 and pDEST15-rDHFR respectively. Each recombinant clone was then transformed into XL-10 – Gold Ultra competent cells (Stratagene). Positive clones were selected for on LB Agar plates containing 50µg/ml ampicillin. Plasmid

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2.3.9 Site Directed Mutagenesis

All site directed mutagenesis was performed using QuickChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies). Primers used were designed as per manufacturer's instructions and are listed in Table 2.1. Both primers contain the required mutation and anneal to the same sequence on opposite strands of the plasmid. The following reagents were added to a eppendorf tube; 5µl 10X reaction buffer, 10ng plasmid DNA, 125ng of forward primer, 125ng of reverse primer, 1µl dNTP mix, 3µl of QuikSolution and H_2O in a total volume of 50µl. 2.5U of *Pfu* Ultra High Fidelity DNA polymerase was also added to the reaction. Samples were then placed on a thermo cycler under the following parameters:

95 ^oC	1 minute	I cycle
95 ^oC	50 seconds	
60^oC	50 seconds	18 cycles
68^oC	1min/kb of plasmid	
68^oC	7 minutes	1 cycle

Parental plasmid DNA was then removed by *Dpn* I digestion. 10U of the enzyme was added to each reaction and incubated at 37^{o}C for 1 hour. *Dpn* I treated samples were then transformed into XL-10 Gold cells and transformation reactions were spread on LB Agar plates containing 50µg/ml ampicillin and incubated overnight at 37^{o}C. Single colonies were picked from transformation plate and an overnight culture grown at 37^{o}C, 220rpm in LB broth containing 50µg/ml ampicillin. Plasmid DNA was isolated from overnight cultures and mutagenesis was confirmed by Sanger sequencing.

2.4 Protein Isolation and Analysis Methods

2.4.1a Protein Isolation from mammalian cells

Cells were trypsinized and centrifuged at 500 x g for 5 minutes at 4^{o}C. The supernatant was removed and discarded. The cell pellet was washed with 1ml of sterile PBS and then resuspended in 500µl of protein lysis buffer. Samples were left on ice for 1 hour, vortexing every 10 minutes. The samples were then centrifuged at max speed for 10 min at 4^{o}C. The supernatant was removed and stored at -20^{o}C, the pellet was discarded.

2.4.1b Production of Recombinant Human DHFR and DHFRL1 protein

Plasmid DNA from GST expression clone was transformed into BL21-AI™ One Shot® Cells (Invitrogen) and cultured in LB Broth containing 100µg/ml ampicillin at 37^{o}C, 220rpm until and OD_{600} of 0.6 was reached. This culture was then used to inoculate fresh LB Broth and grown at 37^{o}C, 220rpm until an OD_{600} of 0.4 was reached. Each culture was then split in two and L-arbinose was added to one culture at a final concentration of 0.2% to induce expression. Aliquots of both cultures were taken at various time points up to 24 hours. Cells were lysed and separated into soluble and insoluble fractions by freezing samples in ice-cold isopropanol and thawing at 42^{o}C three times. Lysed cells were then centrifuged at 16,000 x g, 4^{o}C, for 2 min to pellet insoluble proteins. Expression of protein of interest was determined by SDS-PAGE analysis. Once expression was confirmed, the soluble fraction was then added to glutathione agarose and incubated overnight at 4^{o}C, with gentle mixing. The glutathione agarose was washed with PBS to remove unbound protein. Bound GST –DHFRL1 protein was eluted with elution buffer (50mM Tris-HCL, pH 8.0, 35mM glutathione). All purification fractions were analyzed by SDS PAGE. The eluted fractions were heated to 70^{o}C for 5 min and then digested with TEV protease (Invitrogen) at 30^{o}C overnight in TEV digestion buffer (50mM Tris-HCl, pH 8.0, 0.5mM EDTA, and 1mM DTT). The digested proteins were then added to glutathione agarose and incubated overnight at 4^{o}C, with gentle mixing. The purified protein was collected as the flow through fraction and stored in 20% v/v glycerol. Fractions were again analyzed by SDS-PAGE.

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2.4.2 Estimation of Protein Concentration

Protein concentration was determined by Bicinchoninic Acid (BCA) Assay. A range of standards from 25 - 2000µg of Bovine Serum Albumin (BSA) were made in sterile H_2O. The working reagent was made up to a ratio of 1:50 of reagent A to reagent B. A total volume of 10µl of either standard or sample was added to a 96 well micro plate. The working reagent, 100µl, was then added to each well. The plate was incubated at 37^{o}C for 30 min. A_{562} nm was then measured using Tecan i600 spectrophotometer. A standard curve was then established for the BSA samples and this was used to calculate the concentration of unknown protein samples.

2.4.3 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE gels were made up as follows; 3.3ml (10%) acrylamide 30%, 2.5ml 1.5M Tris pH8.8, 100µl 10% SDS, 100µl 10% APS, 4µl TEMED the volume was brought up to a total of 10ml with dH_2O. This resolving gel was used to fill the plates 2/3 of the way the remainder was filled using the following stacking gel. Acrylamide 30% 830µl, 1M Tris pH6.8 630µl, 10% SDS 50µl, 10% APS 50µl, temed 5µl the volume was brought up to a total of 5ml using dH_2O. Protein samples were mixed with 4X LDS sample buffer and heated to 90^{o}C for 5min before

loading onto a gel. The electrophoresis buffer used was 1x Tris-Glycine buffer. Samples were electrophoresised for approx 45 min at 140 volts. The gel was then stained for 1 hour at room temperature in Coomassie Blue stained and destained at room temperature using 10% acetic acid.

2.4.4 Western Blot

An SDS-PAGE gel was run as described in protocol 2.4.3. Instead of staining with Coomassie Blue the gel was transferred onto nitrocellulose membrane using either wet transfer in a Bio-Rad tank or semi-dry transfer using iBlot (Invitrogen). A wet transfer was done in 1X transfer buffer at 30 volts for 90 min on ice. A semi-dry transfer was carried out by incubating the gel and membrane in the iBlot for 12 min. The membrane was stained with Ponceau S to determine successful transfer and then washed in dH_2O. The membrane was blocked in 5% milk Marvel solution at room temperature for 1 hour. Blot was then washed 3 times at 5 minutes each in TBS-1% Tween. Primary antibody (made up in blocking buffer) was added at a 1:1000 dilution and incubated at 4^oC overnight. Blot was then washed 3 times at 5 minutes each in TBS-1% Tween. Secondary antibody (made up in blocking buffer) was added at a 1:100,000 dilutions and incubated at room temperature for 1 hour. Blot was then washed 3 times at 5 minutes each in TBS-1% Tween. The membrane was the incubated at room temperature for 5 min in Super Signal west femto before image was taken using Gene Genome instrument.

2.4.5 Measurement of enzyme activity

Enzyme activity was tested using a Dihydrofolate Assay Kit (Sigma Cat No. CS03040-1KT). The assay was performed according to manufacturer's instructions. Assays were performed at room temperature in a 96 well flat bottom micro plates, using a Tecan i600 spectrophotometer. The absorbance was read at 340nm using the kinetic mode reading every 30 seconds for 10 minutes. One unit of enzyme is defined as the amount which reduces 1μmol of dihydrofolate per minute based on the molar extinction coefficient of 12,300 $M^{-1}cm^{-1}$ at 340nm.

The K_m values for dihydrofolate were determined in the presence of 50μM NADPH by varying the dihydrofolate concentration from 0.1μM to 2μM and 2.5μM to 700μM. The K_m values for NADPH were determined by varying the concentration of NADPH from 2.5μM to 150μM in the presence of 60μM dihydrofolate. Reactions were initiated by the addition of

substrate. The data were fitted to the Michaelis – Menten equation using Enzfitter software (Biosoft, Cambridge, UK).

2.5 RNA Electrophoretic Mobility Shift Assay (REMSA)

2.5.1 *In Vitro* Transcription

Plasmid DNA as template for this reaction must be first linerized. PCR products can also be used as template provided T7 promoter is incorporated into the forward primer. The following components were added to a 40µl total reaction; 10X transcription buffer, 20X ribonucleotide solution mix, 1µg of template, 20X HMV mix, T7 RNA polymerase (1000U) and RNase free H_2O. Reactions were incubated at 42^oC 4 hours. Results were analyzed on a 1% agarose gel.

2.5.2 RNA 3' end Biotinylation

RNA was transcribed as outline in Section 2.5.1. The RNA was then heated at 85^oC for 5 minutes and placed on ice. The following components were added to a 30µl total reaction; 10X RNA Ligase Reaction Buffer, RNase inhibitor (40U), template RNA (50pmol), Biotinylated Cytidine (Bis) phosphate (1nmol), T4 RNA Ligase (40U), 15% PEG and nuclease free H_2O. The reactions were incubated at 16^oC overnight and then 70µl of H_2O was added. Chloroform: isoamyl alcohol at a volume of 100µl was added and the reaction was then vortexed briefly and centrifuged at 13,000 x g for 3 minutes. The top phase was removed and transferred to a new tube. The following were then added to the reaction; 10µl %M NaCl, 1µl glycogen and 300µl ice-cold 100% ethanol. The reactions were precipated at -20^oC for ≥1 hour. The samples were then centrifuged at 13,000 x g, 4^oC for 15 minutes. The supernatant was removed and discarded and the pellet was washed with 300µl ice cold 70% ethanol. The ethanol was removed and the pellet was allowed to air dry. The pellet was resuspended in 20µl of nuclease free H_2O and stored at -20^oC.

2.5.3 REMSA

Protein binding reaction

Each reaction (total volume, 20 µl) contained biotinylated RNA probes (2nM), DHFR/DHFRL1 protein (100 pmol), 2µg of yeast tRNA (nonspecific competitor, Thermo Scientific) in binding buffer (100mM HEPES, 200mM KCl, 10mM $MgCl_2$, 10mM DTT), 30% (v/v) glycerol. In samples with specific competitor was added 1uM of unlabelled DHFR/DHFRL1 RNA. Labelled RNA and protein was incubated at room temperature for 10 minutes before adding to the reaction. Following the addition of 100µg of heparin (nonspecific competitor) samples were incubated at 65^{o}C for 30 minutes and then at room temperature for a further 10 minutes.

REMSA

The entire reaction sample was resolved on a 4% non-denaturing polyacrylamide (59:1) gel (8 x 8 x 0.1 cm) in 0.5% TBE buffer for 1 hour at 100 V on ice. Gel was pre-run 40 minutes and samples were loaded without loading buffer. Nucleic acids were wet transferred on nylon membrane 45 minutes at 350 mA. Membrane was cross-linked for 1 minute at 120 mJ/cm2 using a UV-light cross-linker instrument. Chemiluminescent bands were detected by LightShift® Chemiluminescent EMSA Kit (Thermo Scientific) on a Gene Gnome instrument.

2.6 *In Vitro* Translation Assay

The following components were added to the reaction; rabbit lysate (10µl), potassium acetate (66µM), magnesium acetate (0.5mM), amino acid – methionine (0.5µl), amino acid – leucine (0.5µl), RNA (2nM) and protein (100pmol). Reactions were incubated at 37^{o}C for 90 minutes. Results were analysed by Western blot as outlined in 2.4.4.

2.7 Mitochondria Isolation

Mitochondria were isolated using Q-proteome Mitochondria Isolation Kit (Qiagen) using a modified protocol. Cell suspension was centrifuged at 500 x g for 10 min at 4^{o}C and the supernatant discarded. The pellet was then washed using 1ml 0.9% NaCl and then re-suspended in 200µl of lysis buffer. Lysates were incubated at 4^{o}C for 10 min on a shaker before being centrifuged at 1000 x g for 10 min at 4^{o}C. The supernatant was removed and stored at -20^{o}C as it contained the cytoplasmic fraction. The pellet was resuspended in 500µl ice-cold disruption buffer and passes through a blunt end needle and syringe 10 times to complete disruption. Lysates were then centrifuged at 1000 x g for 10 min at 4^{o}C. The supernatant was transferred to a new tube and the pellet was discarded. The supernatant was centrifuged at 10,000 x g for 10min at 4^{o}C. The supernatant was removed and discarded; the pellet contains the mitochondria fraction. The pellet was then re-suspended in 50µl of protein lysis buffer.

2.8 Acetone Precipitation of proteins

Low yield proteins were concentrated by acetone precipitation. Four volumes of ice-cold acetone was added to the protein fraction and incubated on ice for 15 min. Samples were then centrifuged for 10 min at 12,000 x g at 4^{o}C. The supernatant was discarded and the pellet washed with 1ml 80% acetone. The pellet was allowed to air dry and re-suspended in 50µl protein lysis buffer.

2.9 *in vitro* SUMOylation assay

Potential sumoylation of DHFRL1 by SUMO-1 protein was tested using SUMOlink™ SUMO-1 kit (Active Motif) as per manufacturer's instructions. The assay components were incubated with 0.5µg/µl of DHFRL1 protein for 3 hours at 30^{o}C. The reaction was stopped by adding equal volume of SDS loading buffer. Results were analyzed by western blot and samples were stored at -20^{o}C. A reaction containing DHFR as the protein of interest was set up as a positive control. Negative control reactions included a sumo-1 mutant protein which cannot sumoylate target protein.

2.10 Antibody Production Methods

2.10a Mouse Immunization

A peptide from the N terminal region of the DHFRL1 protein was designed and synthesized by Cambridge Bioscience. The peptide sequence was FLLLNCIVA and was conjugated to a KLH molecule at the N terminal. This peptide was used to immunize three Balbc mice (200µl peptide, 400µl PBS and 600µl Freund's incomplete adjuvant). Blood samples were taken from each of the immunized mice and also from an unimmunized mouse 21 days after immunization. Blood samples were incubated at 4^{o}C overnight to allow the blood to clot. Blood was centrifuged at 10,000 x g for 10 minutes to isolate serum. A serial dilution of the serum ranging from 1:100 – 1:1,000,000 was then prepared in PBS 0.5% tween. Mice were given up to 5 booster shots to increase antibody production.

2.10b Enzyme Linked Immunosorbent Assay (ELISA) to measure serum titre

The wells of a 96 well plate were coated with 1µg/ml of either DHFR or DHFRL1 protein in PBS in a total volume of 100µl and incubated overnight at 4^{o}C. A section of the plate was left uncoated. All of the plate was then blocked in 5% milk solution at room temperature for 2 hours. There was no wash step between coating and blocking the plate. After blocking the plate was washed three times in PBS. The serum dilutions were then added to the plate and incubated at 37^{o}C for 1 hour. The plate was then washed three times in PBST and once in PBS. Anti-mouse HRP secondary antibody (1:2000 dilutions) was added to the plate and incubated at 37^{o}C for 1 hour. The plate was then washed three times in PBST and once in PBS. TMB (Tetramethylbendzidine) substrate in a total volume of 100µl was added to the plate and incubated at room temperature for approximately 15 minutes (until the wells go blue). At this stage the plate was wrapped in tinfoil to protect it from the light. Stop solution (10% HCL) in a total volume of 100µl was added to each well. The plate was then read at 450nm using Tecan i600 spectrophotometer. Results were plotted as log of serum dilutions versus OD 450nm.

Table 2.1 Primer Sequences

Primer Name	Sequence
Protein Expression Experiment	
Human GST DHFR Forward	5’GGGGACAAGTTTGTACAAAAAAGCAGGC TTCGAAAATCTGTACTTCCAGGGGATGGTT GGTTCGCTAAACTG 3’
Human GST DHFR Reverse	5’GGGGACAAGTTTGTACAAAAAAGCAGGC TTCGAAAATCTGTACTTCCAGGGGATGTTTC TTTTGCTAAACTG 3’
Human GST DHFRL1 Forward	5’GGGGACAAGTTTGTACAAAAAAGCAGGC TTCGAAAATCTGTACTTCCAGGGGATGTTTC TTTTGCTAAACTG 3’
Human GST DHFRL1 Reverse	5’GGGGACCACTTTGTACAACAAAGCTGGGT CCTAATCATCCTTCTCACATA 3’

	AACTTCTGGGAA 3’	
Adapter Forward	5’GGGGACAAGTTTGTACAAAAAAGCAGGC T 3’	
Adapter Reverse	5’GGGGACCACTTTGTACAAGAAAGCTGGGT 3’	
E-coli Complementation Study		
DHFR Forward	5’GATGGTTGGTTCGCTAAACT 3’	
DHFR Reverse	5’TTAATCATTCTTCTCATATCGTTC 3’	
DHFRL1 Forward	5’GATGTTTCTTTTGCTAAACTGC 3’	
DHFRL1 Reverse	5’TTAATCATCCTTCTCACATACTTC 3’	
REMSA		
T7 Forward	5’TAATACGACTCACTATAGGG 3’	
UPL (RT-qPCR)		
Human DHFR Forward	5’ GGGGGAAAGCTGGAGTATTG 3’	Probe #24
Human DHFR Reverse	5’ ACTATGTTCCGCCCACACAC 3'	
Human DHFRL1 T1 Forward	5’ TCCAGAAGCGTCTCATTCAG 3’	Probe #57
Human DHFRL1 T1 Reverse	5’ TCTCAGCGGGGACAATGCT 3’	
Human DHFRL1 T2 Forward	5’ CGGACCTTAGAAAGTCACACATC 3’	Probe # 89
Human DHFRL1 T2 Reverse	5’ GCGAAATTCCCTTCTTCAAA 3’	
Human GUS Forward	5’ CGCCCTGCCTATCTGTATTC 3’	Probe # 57
Human GUS Reverse	5’ TCCCCACAGGGAGTGTCTAG 3’	
Human β-actin Forward	5’ CCAACCGCGAGAAGATGA 3’	Probe # 64
Human β-actin Reverse	5’ TCCATCACGATGCCAGTG 3’	
Mouse DHFR Forward	5’ AACCGGAATTGGCAAGTAAA 3’	

Mouse DHFR Reverse	5’ CCTGGTTGATTCATGGCTTC 3’	Probe # 16
Mouse DHFRLS Forward	5’ CCAGGATGGTCCGATTTTT 3’	
Mouse DHFRLS Reverse	5’ GCGAAGAAGCACTTCAATGG 3’	Probe # 32
Mouse β-actin Forward	5’ CTAAGGCCAACCGTGAAAAG 3’	
Mouse β-actin Reverse	5’ ACCAGAGGCATACAGGGACA 3’	Probe # 64
Rat DHFR Forward	5’ AAAGTGGACATGGTCTGGGTA 3’	
Rat DHFR Reverse	5’ CTGGCTGATTCATGGCTTC 3’	Probe # 16
Rat DHFRLS Forward	5’GGACATAGTTTGGATAATCAGAG3’	
Rat DHFRLS Reverse	5’ ATGATCCTTGTCACAAAAAG 3’	
Rat Ywhaz Forward	5’ CTACCGCTACTTGGCTGAGG 3’	
Rat Ywhaz Reverse	5’ TGTGACTGGTCCACAATTCC 3’	Probe # 9
Rat β-actin Forward	5’ CCCGCGAGTACAACCTTCT 3’	
Rat βactin Reverse	5’ CGTCATCCATGGCGAACT 3’	Probe # 17
CHO β-actin Forward	5’ GCTATGAGCTGCCTGATGG 3’	
CHO β-actin Reverse	5’ GGCTGGAAAAGAGCCTCA 3’	Probe # 64

Site Directed Mutagenesis

Human DHFRL1 Forward	5’ GCAGGCACCATGGTTGGTTCG 3’
Human DHFRL1 Reverse	5’ CGAACCAACCATGGTGCCTGC 3’

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CHAPTER 3

Assessment of enzyme activity of DHFRL1

3.1 Introduction

Dihydrofolate reductase (DHFR) is an enzyme which reduces dihydrofolate into tetrahydrofolate in the presence of NADPH. This reaction is essential for the proliferation of the cell as tetrahydrofolate is the biologically active form of folate and as such it is required for the *de novo* synthesis of purines and thymidylate along with glycine, methionine and serine.[93] DHFR is also the only enzyme known to reduce folic acid, the synthetic form of folate, found in all fortified foods. DHFR enzyme can also catalyze the reduction of dihydrobiopterin to tetrahydrobiopterin, a function which has recently been shown to be important in the NOS salvage pathway in endothelial cells.[94]

Due to its importance within the cell DHFR has been well characterized over the years and the amino acids required not only for catalytic activity but specifically folate and NADPH binding have been identified. These amino acids are all highly conserved in DHFRL1 with just 3 notable exceptions at positions 24 (W24R), 54 (K54R) and 81 (V81I). Sequence alignment is shown in Figure 3.1 and predicted tertiary protein structure for each protein is shown in Figure 3.2. The substitution of W24R would appear to be significant as previous studies have shown that a tryptophan at position 24 of DHFR is important for substrate binding. A site-directed mutagenesis study carried out by Beard *et al* (1990) showed that a W24F mutant of DHFR had a 50% decrease in stability and 48% decrease in efficiency under intracellular conditions.[95] An earlier study carried out by Thillet *et al* (1998) on mouse DHFR showed that a W24R mutant resulted in a much lower binding efficiency for dihydrofolate but not NADPH.[96] However a 2005 study carried out by Cody *et al* showed that there are significant differences between human and mouse DHFR active sites.[97]

Assessment of dihydrofolate reductase activity by DHFRL1 was carried out using the following approach:

- Creation of DHFRL1 expression clones.
- Over expression of DHFRL1 in DHFR negative cells both bacterial and mammalian.
- Detailed enzyme kinetic analysis of purified recombinant DHFRL1 protein.

RESULTS

3.2 Generation of DHFR and DHFRL1 expression clones.

The DHFR and DHFRL1 expression clones constructed for both the production of recombinant protein and the complementation studies in mammalian cells were created using the Gateway® cloning system. The advantage of the Gateway® system over conventional cloning methods is once the entry clone has been created the gene of interest can then be shuttled into various destination vectors depending on the downstream application. An overview of Gateway® cloning is shown in Figure 3.3.

Mammalian Expression Clones

The DHFR/DHFRL1 mammalian expression clones were created as outlined in Section 2.3.7a. The destination vector pcDNA3.2/V5 was used as it contains a CMV promoter which is essential for successful expression in mammalian cells. Plasmid DNA was purified from the entry clone (Figure 3.4) and recombined into the pcDNA3.2/V5 vector. This construct was then transformed into DH5α cells and positive clones were confirmed by restriction digest with *Xba* I which linearizes DHFRL1 but gives two bands for DHFR (Figure 3.5) and Sanger sequencing.

Bacterial GST Expression Clones

For the production of recombinant DHFR/DHFRL1 protein a GST tagged clone was created for each gene as outlined in 2.3.7a. An entry clone for both DHFR and DHFRL1 was created by cloning PCR product amplified from HEK 293 cDNA, (Figure 3.6) and recombined into a pDONR™ 211 vector. Plasmid DNA from this construct was then recombined into pDEST™ 15 vector which contains a GST tag at the N terminus. Positive clones were again confirmed by restriction digest using *EcoR*I which cuts the empty vector at 2 sites and the DHFR/DHFRL1 clones at 3 sites, (Figure 3.7) and Sanger sequencing.

Bacterial Complementation Clones

The DHFR/DHFRL1 expression clones used for the complementation study in a bacterial system were constructed using the traditional TA cloning system outlined in 2.3.7b. The ORF for each gene was PCR amplified using plasmid DNA from pDEST15-rDHFRL1 and pDEST15-rDHFR respectively, (Figure3.8). The PCR template was then ligated into pCR2.1 vector and the ligation reaction was transformed into XL-10 Gold Ultra Competent Cells. Positive clones were confirmed by restriction digest and Sanger sequencing.

3.3 Complementation of a dhfr negative phenotype by DHFRL1 in a bacterial system.

Escherichia coli D3-157 cells, first isolated by Singer *et al* in 1985, do not have any DHFR function and require thymidine supplementation in the media in order to grow.[98] These cells are also streptomycin resistant so this antibiotic is also added to the media to act as a selective marker. *E-coli* D3-157 cells were transformed with pCR2.1 with DHFRL1 insert and allowed to grow in thymidine supplemented media until OD of 0.4 was reached. A 1ml aliquot of this culture was then used to inoculate 20ml of the following media:

i. LB Broth containing Streptomycin, Ampicillin, IPTG and Thymidine.
ii. LB Broth containing Thymidine and Streptomycin.
iii. LB Broth containing Streptomycin, Ampicillin an IPTG.
iv. LB Broth containing Streptomycin and Ampicillin.
v. LB Broth containing Streptomycin, Ampicillin and Thymidine.

Positive control used in this experiment was cells transformed with pCR2.1 with DHFR insert, while untransformed cells were used as a negative control. Cells were also transformed with an empty pCR2.1 vector to ensure the results obtained were not caused by the vector itself. IPTG was added to induce expression. Growth was measured in a UV spectrophotometer at 600nm. A 1ml aliquot of each culture was taken at following time points: 0, 2, 4, 6, 24, 30, 48, 72 hours and absorbance at 600nm was measured. The resulting data points were plotted and a growth curve for each culture was established. Results are shown in Figure 3.9.

Both positive and negative controls grew as expected. The original strain only grew in media containing streptomycin and thymidine. The DHFR transformed cells grew in all media containing thymidine and also the media containing streptomycin, ampicillin and IPTG i.e. the unsupplemented media. Some growth was also observed in media containing streptomycin and ampicillin only. Cells transformed with DHFRL1, behaving in a similar fashion to the positive control, grew in media supplemented with thymidine and also media without thymidine. Once again there was some growth in the media containing streptomycin and ampicillin.

Cells were not expected to grow in the media containing streptomycin and ampicillin only as there is no thymidine present or no IPTG present to induce the production of DHFR/DHFRL1. For the untransformed cells and cells transformed with empty vector no growth was observed, therefore the low levels of growth observed for cells transformed with either DHFR or DHFRL1 maybe be due to the presence of a leaky promoter which expressed low levels of DHFR/DHFRL1 despite the absence of the inducer substrate IPTG

The conclusion drawn from this experiment was that DHFRL1 harboured enough dihydrofolate reductase activity to complement a dhfr negative phenotype in a bacterial system.

3.4 Complementation of dhfr negative phenotype by DHFRL1 in a mammalian system.

Having established complementation of the phenotype in a bacterial system the experiment was then repeated in mammalian cells. CHO DG44 cells are a Chinese hamster ovary cell line that has undergone mutagenesis so that they do not have a functional DHFR.[99] Like the *E-coli* D3-157 cells these cells require thymidine in the media in order to grow. As they are glycine auxotroph these cells also require a hypoxthaine supplement in order to survive.

These cells were transfected with an empty pcDNA3.2/V5 vector, a DHFR pcDNA3.2/V5 construct or a DHFRL1 pcDNA3.2/V5 construct. The cells were allowed to grow in complete media for 48 hours before 500μg/ml of G418 (a neomycin derivative) was added to the media to positively select for transfected cells. These cells were maintained in selection media for approximately 14 days to ensure that only transfected cells remained in the culture. At this point

cells were switched to media without any thymidine or hypoxthaine. Cells were counted by haemocytometer using trypan blue at Day 1, Day 5 and Day 12, (Section 2.2.2). Results are shown in Figure 3.10.

Cells transfected with the empty pcDNA3.2/V5 vector, which acted as the negative control, did not survive beyond Day 1 and could not grow without supplements. Cells transfected with DHFR pcDNA3.2/V5, the positive control, showed some cell death at Day 5 however they had fully recovered by Day 12 and were able to grow without thymidine or hypoxthaine. The cells transfected with DHFRL1 pcDNA3.2/V5 once again behaved in a similar fashion to the positive control. A certain amount of cell death was observed at Day 5 however by Day 12 the cells had recovered although they did not grow as quickly or efficiently as the DHFR transfected cells.

Protein was harvested from the cells after 12 days in complementation media and enzyme activity was measured (Section 2.4.5). Results are shown in Figure 3.11. Cells transfected with DHFRL1 showed a 26% lower specific activity when compared to cells transfected with DHFR i.e. DHFRL1 cells had 26% less efficient dihydrofolate reductase activity which may explain why the DHFRL1 transfected cells had a lower cell number on day 12. The results from this experiment confirmed what was observed in the bacterial complementation study i.e. that DHFRL1 does harbour some dihydrofolate reductase enzyme activity.

3.5 Production of recombinant DHFRL1 protein

In order to carry out a more detailed enzyme kinetic analysis of DHFRL1 a purified recombinant protein was first produced. Initially DHFRL1 was cloned into Gateway vector pDEST 17 which contains a HIS tag at the N terminus. However attempts to purify the protein were unsuccessful as the protein of interest was only present in the insoluble fraction after cell lysis, (Figure 3.12). DHFRL1 was then cloned into Gateway Cloning vector pDEST 15 (Section2.4.1) which contains a GST tag at the N terminus. A TEV (tobacco etch virus) recognition site was included upstream of the DHFRL1 protein but downstream of the GST tag at this stage. The vector construct was then transformed into DH5α *E-coli* cells which can be used to maintain a bacterial stock of the clone. Plasmid was isolated from overnight culture of the DH5α cells (Section 2.3.2) and used to transform Bl 21-A1 *E-coli* cells. This cell line has been engineered to ensure optimum

expression of protein of interest by exploiting the use of an inducer system. Transformed Bl 21-A1 cells were induced using 0.2% L-arbinose and cultures were grown overnight at 37 ^{o}C. Protein was harvested from overnight cultures and fractionated into soluble and insoluble fractions by multiple freeze thaw cycles. SDS-PAGE analysis was carried out to ensure:

a) Protein of interest was expressed
b) Protein of interest was contained in the soluble fraction

Results are shown in Figure 3.13 and clearly show that the protein of interest is both strongly expressed and present in the soluble fraction allowing purification to take place.

Having established that the protein of interest was present in the soluble fraction, the GST tagged protein was purified by binding the GST tag to glutathione agarose. The purified protein was eluted using 35mM glutathione, which competes with the GST to bind to the glutathione agarose. The protein purification fractions were analyzed by SDS-PAGE. Results are shown in Figure 3.14 depict a single band of the correct size in the elution fraction indicating a purified protein is present.

The final step of the process was to cleave the GST tag from the DHFRL1 protein. This was done using a TEV cleavage enzyme which cuts the protein at the recognition site incorporated in at the cloning stage. GST-DHFRL1 protein was treated with 5U of TEV enzyme and incubated overnight at 30^{o}C. The sample was then passed through the glutathione agarose column once more. The DHFRL1 was released in the wash steps while the GST remained bound to the column. The fractions were analyzed by SDS-PAGE. Results are shown in Figure 3.15. Purified recombinant DHFR protein was also produced using this method.

3.6 Enzyme kinetic analysis of purified DHFRL1 protein

The complementation studies outlined in Sections 3.2 and 3.3 confirmed that DHFRL1 does harbour some dihydrofolate reductase activity. A more detailed enzyme kinetic analysis was then carried out on purified recombinant DHFRL1 protein and compared to recombinant DHFR protein produced in the same way. The following kinetic measurements were determined:

1. **Specific Activity** – the amount of substrate used per min per mg of protein i.e. how efficient the enzyme is at breaking down the substrate.
2. **Vmax** – maximum velocity of the enzyme. This measures the amount of time it takes for all the enzymes active sites to be bound.
3. **Km** – Michealis Menten constant measures how much substrate is needed to reach half Vmax. This tells us the affinity the enzyme has for the substrate or how well it binds the substrate. The higher the Km the lower the affinity.
4. **Kcat** – the catalytic constant of the enzyme or the turnover rate of the enzyme. This is the time required by the enzyme to "turn over" one substrate molecule.
5. **Kcat/Km** – this represents the catalytic efficiency of the enzyme. The greater the value of kcat/km the faster and more efficiently the substrate is converted into product

Both Vmax and Km were calculated not only for the substrate dihydrofolic acid but also for the co-factor NADPH. Kcat measurements were taken for the substrate dihydrofolic acid and calculated using the formula Vmax / [E]total [100] (where concentration of enzyme was expressed as μmol).

The specific activity measurements were based on the molar extinction coefficient of 12,300 $M^{-1}cm^{-1}$ at 340nm and showed that DHFR was more efficient at breaking down the substrate than DHFRL1. The measurement for DHFR and DHFRL1 were 6.1 and 4.3 μmol/min/mg respectively. The Kcat/Km measurement is also an indicator of catalytic efficiency and as with the specific activity measurement DHFRL1 showed a Kcat/Km value of 32.61 $sec^{-1}\mu M^{-1}$ which is lower than the DHFR value of 62.14 $sec^{-1}\mu M^{-1}$ indicating that DHFRL1 is not as efficient at breaking down the substrate compared to DHFR.

The Km and Vmax values for the co-factor NADPH were determined by keeping the substrate, dihydrofolic acid, constant at 60μM and varying the concentration of NADPH between 2.5 and

150μM. The Km values for NADPH for both DHFR and DHFRL1 are similar at 3.6μM and 3.4μM respectively, indicating that DHFRL1 has an equal affinity with DHFR for the co-factor NADPH. This result is not surprising as the amino acids necessary for NADPH binding are conserved within DHFRL1. The Vmax values for both enzymes were also similar. Results are summarized in Table 3.1.

As predicted by the sequence alignment, (Figure 3.1), the Km values for the substrate, dihydrofolic acid, vary greatly between the two enzymes. The Km and Vmax values were determined over a range of 0.2-2μM, while NADPH was kept constant at 50μM. At this range of substrate concentration DHFRL1 showed a significantly higher Km, approximately 3 fold higher, when compared to DHFR. This result indicates that DHFRL1 has a much lower affinity for the substrate which is the most logical explanation for the lower catalytic efficiency of DHFRL1.

3.7 Discussion

To date it has been assumed that humans have only one dihydrofolate reductase enzyme. The amino acids necessary for DHFR catalytic function are all highly conserved within DHFRL1 predicting that DHFRL1 protein may have enzyme activity. This hypothesis was first proved in the complementation studies carried out in both bacterial and mammalian systems. These experiments confirmed that DHFRL1 does have enzyme activity and can rescue the dhfr negative phenotype. Further enzyme analysis on purified recombinant DHFRL1 protein revealed that while DHFRL1 can efficiently bind the co-factor NADPH it has a much lower affinity for the substrate dihydrofolic acid when compared to purified recombinant DHFR protein.

E-coli are a widely used host system for the production of recombinant proteins and were utilized in this study. This system has a number of advantages including; rapid growth allowing the production of recombinant protein within days, it is relatively inexpensive and is easy to scale up for a higher level of protein production.[101] However as with any method there are limitations. One major challenge with this system is the formation of insoluble inclusion bodies which make the purification of recombinant protein extremely difficult.[102] In this study initial experiments to produce a His tagged recombinant DHFRL1 protein lead to the formation of insoluble protein aggregates, an obstacle which was overcome by switching the affinity tag to the larger GST protein. A second clear disadvantage with using *E-coli* to produce recombinant mammalian protein are the difference between bacterial and mammalian post translational modification systems. For example a number of mammalian proteins undergo glycosylation, the attaching of glycans to the protein molecule, which can be important for protein folding and stability.[103] This process does not occur in bacterial cells, and therefore any protein which endogenously undergoes this process may have slightly different characteristics compared to a recombinant version produced in bacteria. Although there are certain limitations to producing a recombinant protein using an *E-coli* host system, all enzyme kinetic analysis was carried out on DHFR and DHFRL1 protein produced by the same method. While this may not accurately reflect any endogenous post translational modification, it does control for variations due to the method of protein production giving us an accurate comparison of the kinetic properties of the two enzymes.

The possible benefits of having a second dihydrofolate reductase enzyme with a lower affinity for the substrate are as yet not fully understood but it may be the optimal conditions for the sub-cellular location of the enzyme. DHFRL1 has been proven to have a strong presence in the mitochondria. The sub-cellular localization of DHFRL1 is discussed in more detail in chapter 5.

The mitochondria are well established sites for folate metabolism. Their primary function however is to produce the energy required to power the cell and as a result the environmental conditions inside the mitochondria may differ from those in the cytoplasm and an enzyme with a lower affinity for its substrate may be more suited to the mitochondrial environment.

Tetrahydrofolate is the building block for all folate mediated reactions in the mitochondria and as such there are well established folate transport systems in place, namely in the form of mitochondrial folate transporter (MFT), which facilitate entry into the mitochondria.[104] Due to this transport system the mitochondria have other sources of tetrahydrofolate available to it and are not dependent on producing their own, so although DHFRL1 cannot produce tetrahydrofolate as efficiently as DHFR it most likely produces enough to support the reactions required of it. A recent study revealed that DHFRL1 replaces DHFR in the *de novo* thymidylate biosynthesis pathway in the mitochondria,[105] a reaction that is essential for mitochondrial DNA replication. It is possible that the tetrahydrofolate produced by DHFRL1 is required only for this reaction and so an enzyme that produces large quantities of it is not required.

DHFR MVGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQRMTTTSSVEGKQNL 50
DHFRL1 MFLLLNCIVAVSQNMGIGKNGDLPRPPLRNEFRYFQRMTTTSSVEGKQNL 50
*. ********** **********

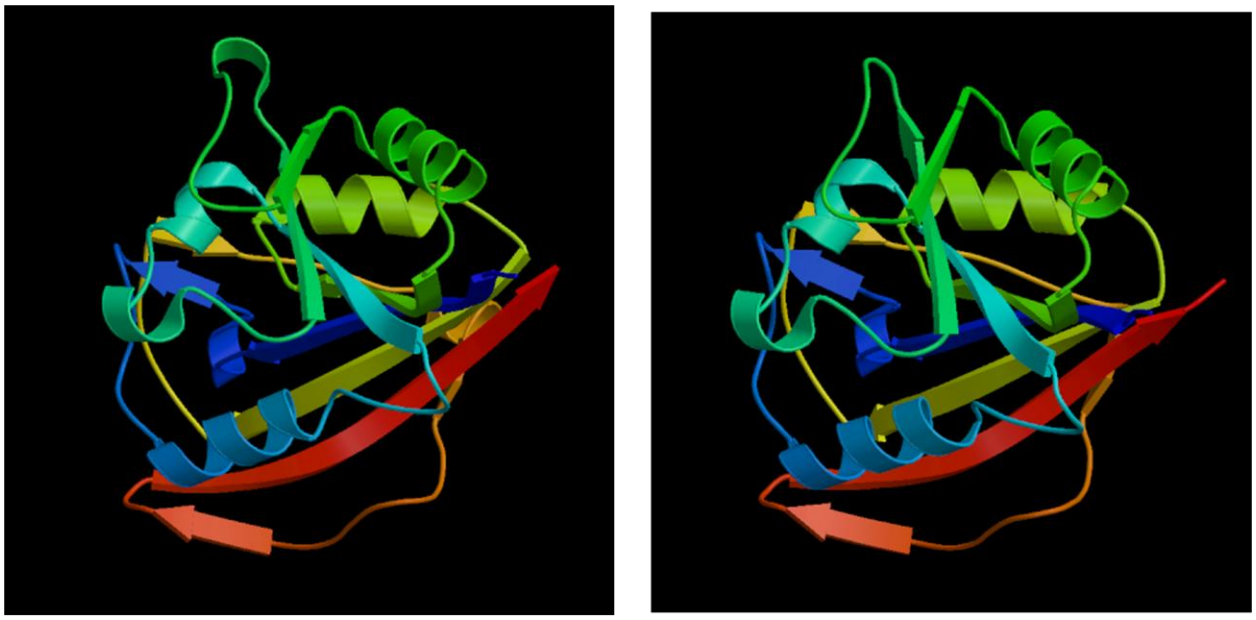
DHFR VIMGKKTWFSIPEKNRPLKGRINLVLSRELKEPPQGAHFLSRSLDDALKL 100
DHFRL1 VIMGRKTWFSIPEKNRPLKDRINLVLSRELKEPPQGAHFLARSLDDALKL 100
****:********** .********** :*********

DHFR TEQPELANKVDMVWIVGGSSVYKEAMNHPGHLKLFVTRIMQDFESDTFFP 150
DHFRL1 TERPELANKVDMIWIVGGSSVYKEAMNHLGHLKLFVTRIMQDFESDTFFS 150
:*******:********** ********** .

DHFR EIDLEKYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND 187
DHFRL1 EIDLEKYKLLPEYPGILSDVQEGKHIKYKFEVCEKDD 187
********** :****** * ******* **:*

Figure 3.1 Sequence alignments of human DHFR and DHFRL1 sequences: Generated using CLUSTAL 2.0.8. The four motifs required for DHFR catalytic activity are highlighted in green (www.bioinf.manchester.ac.uk/dbbrowser/PRINTS/). Specific residues important for folate, NADPH and/or MTX binding are underlined. Specific amino acids important for DHFR mRNA binding are shown in red. Proposed DHFR sumoylation site is shown in blue.

Ref: McEntee G et al (2011). The former annotated human pseudogene dihydrofolate reductase –like 1 (DHFRL1) is expressed and functional. Epub *Proc Natl Acad Sci* www.pnas.org/cgi/doi/10.1073/pnas.1103605108



A **DHFR** **B** **DHFRL1**

Figure 3.2 Predicted tertiary protein structure for human DHFR and DHFRL1. Protein structures were created using software from Protein Structure Prediction Server from Molecular Bioinformatics Centre National Chiao Tung University. **(A)** Shows the predicted structure for DHFR and **(B)** shows the predicted structure for DHFRL1. Both structures are highly similar with all major α helices and β sheets present in both proteins.

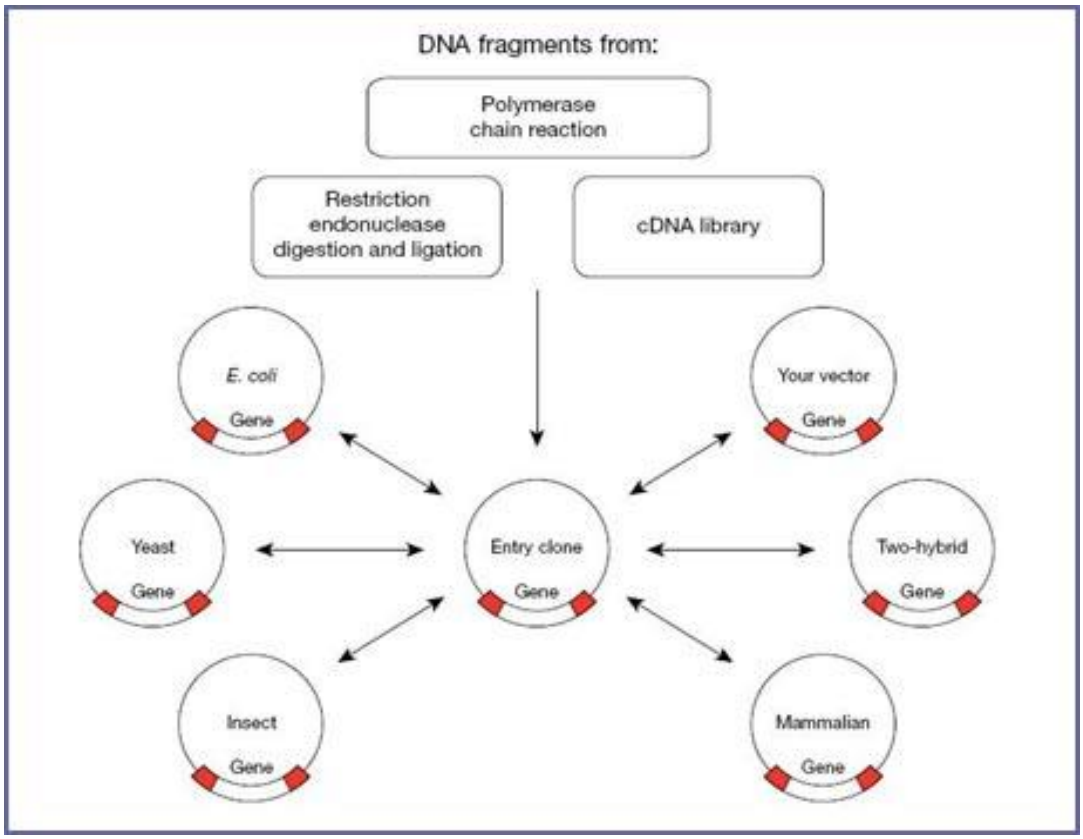
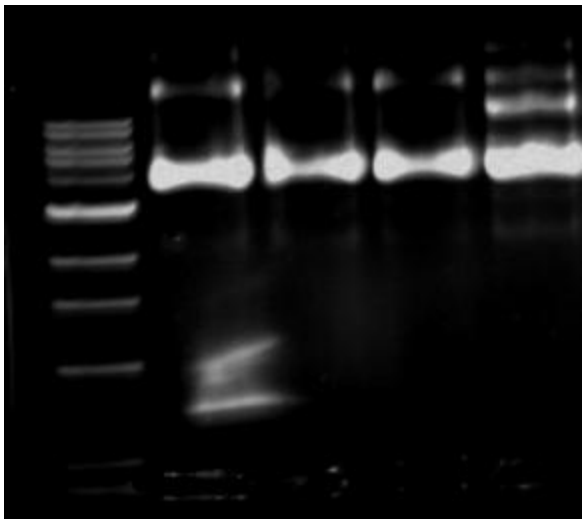
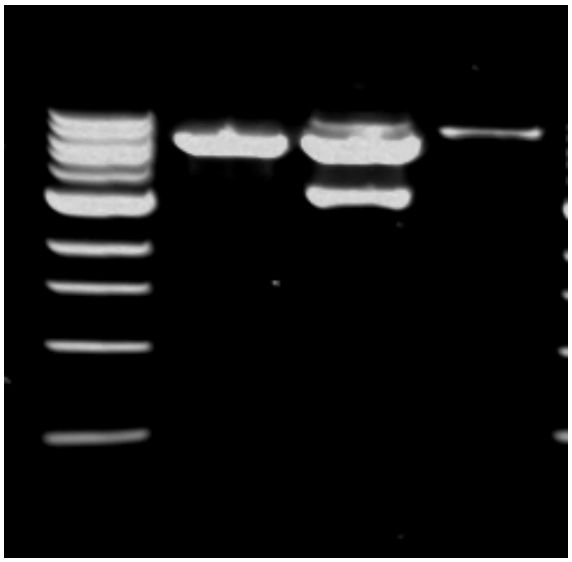


Figure 3.3 Overview of Gateway Cloning. This image was taken from Gateway Technology User Manual (Invitrogen). Gateway cloning allows construction of an entry clone from a number of different sources including PCR templates, cDNA libraries and restriction fragments. Once the entry clone has been created the gene of interest can then be shuttled into various destination vectors depending on the downstream application.



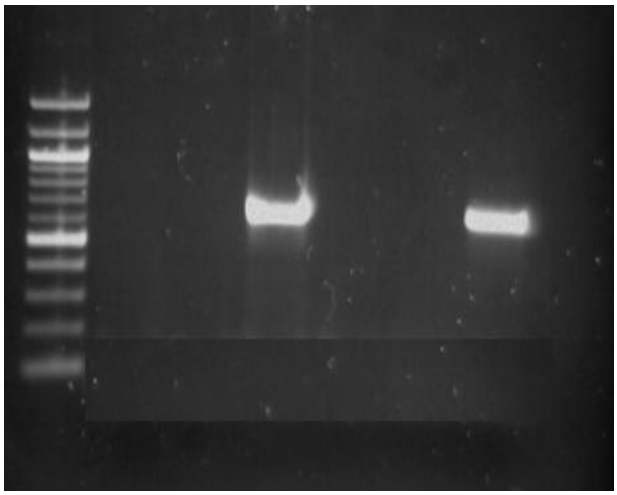
1 2 3 4 5

Figure 3.4 Plasmid DNA purified from DHFR/DHFRL1 entry clones. Lane 1 contains 1kB ladder, **lanes 2+3** show DHFR entry clone plasmid DNA, **lanes 4+5** show DHFRL1 entry clone plasmid DNA.



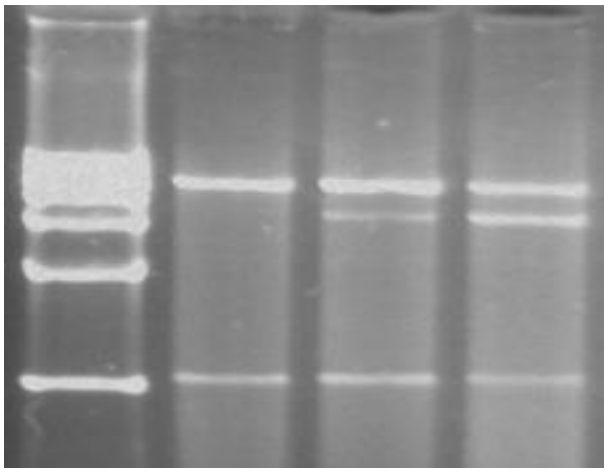
1 2 3 4

Figure 3.5 Restriction digest of pcDNA3.2 +DHFR/DHFRL1 clones. Plasmid DNA from pcDNA3.2 + DHFR/DHFRL1 clones was digested with *Xba* I enzyme which linearizes the DHFRL1 clone and the destination vector but cuts the DHFR clone at two sites giving two bands on the gel. **Lane 1** contains 1kB ladder, **lane 2** shows DHFRL1 clone, **lane 3** shows DHFR clone and **lane 4** shows empty vector.



1 2 3 4 5

Figure 3.6 DHFR/DHFRL1 entry clone PCR. PCR product was amplified from HEK 293 cDNA and used as a template to create an entry clone for the production of recombinant DHFR and DHFRL1 protein. **Lane 1** contains 100bp ladder, **lane 2** PCR negative control, **lane 3** DHFRL1 PCR product (564 bp), **lane 4** PCR negative control and **lane 5** DHFR PCR product (564bp).



1 2 3 4

Figure 3.7 Restriction digest of pDEST 15 +DHFR/DHFRL1 clones. Plasmid DNA from pDEST 15 + DHFR/DHFRL1 clones was treated with EcoRI enzyme which cuts the empty vector at 2 sites and the DHFR/DHFRL1 clones at 3 sites. **Lane 1** contains 1kB ladder, **lane 2** empty vector, **lane 3** pDEST15+ DHFR, and **lane 4** pDEST 15 + DHFRL1.

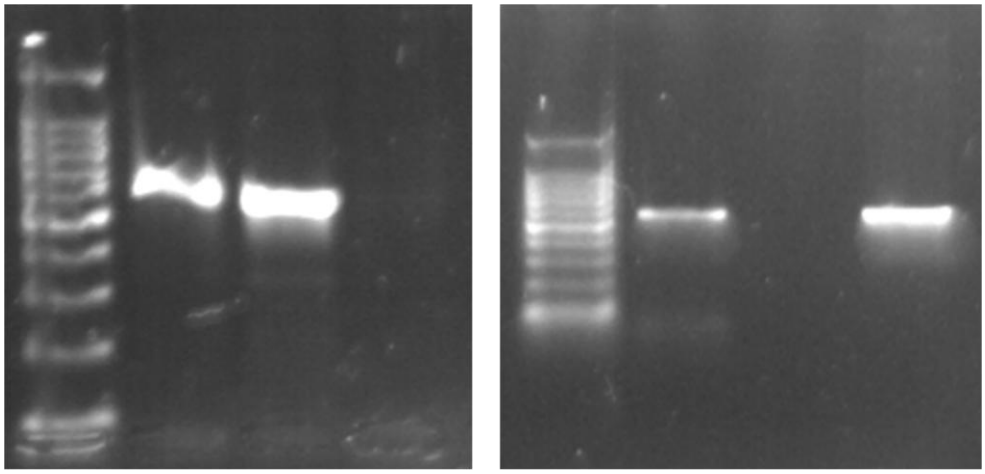
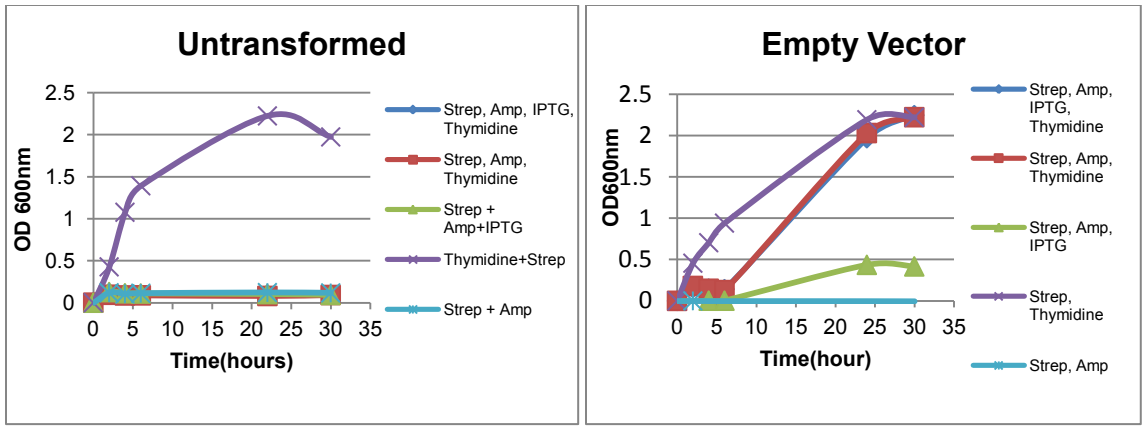
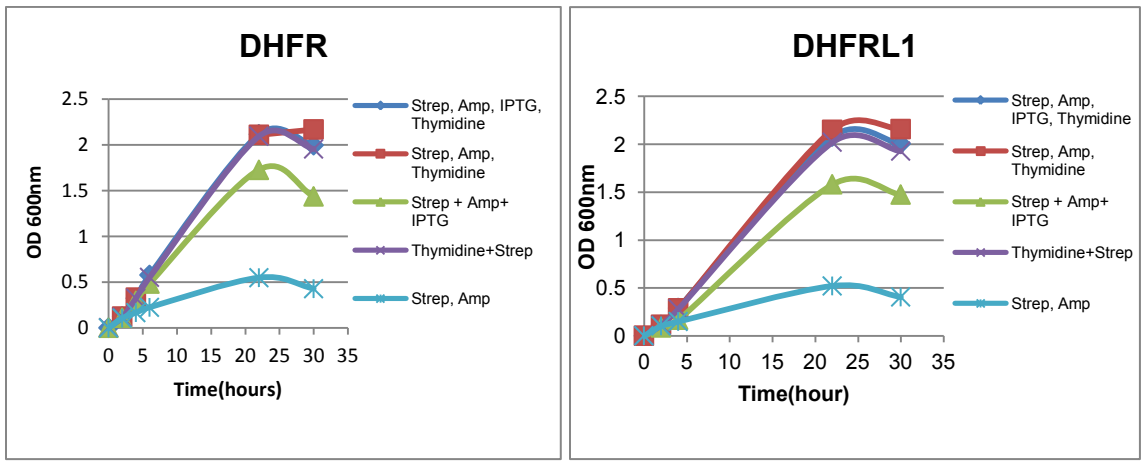


Figure 3.8 DHFR/DHFRL1 complementation study PCR. The ORF for both DHFR and DHFRL1 were PCR amplified from using plasmid DNA from pDEST 15-DHFR and pDEST 15- DHFRL1 respectively. **(A) Lane 1** 100bp ladder, **lane 2+3,** DHFRL1 PCR product (564bp) and **lane 4** PCR negative control **(B)) Lane 1** 100bp ladder, **lane 2,** DHFR PCR product (564bp) **lane 3** PCR negative control and **lane 4** DHFR PCR product (564bp).



A B



C D

Figure 3.9 Complementation of DHFR negative phenotype in a bacterial system. *Escherichia coli* D3-157 cells were transformed with either DHFR or DHFRL1 and grown in media both with and without supplements. Growth was measured at various time points until stationary phase was reached. (**A**) The original strain only grew in media containing thymidine. (**B**) Cells transformed with empty vector grew in supplemented media; there was also some growth in the induced media containing both antibiotics. (**C**) Cells transformed with DHFR grew as expected in media both with and without supplements. (**D**) Cells transformed with DHFRL1 also complemented the phenotype and grew in media both with and without supplements.

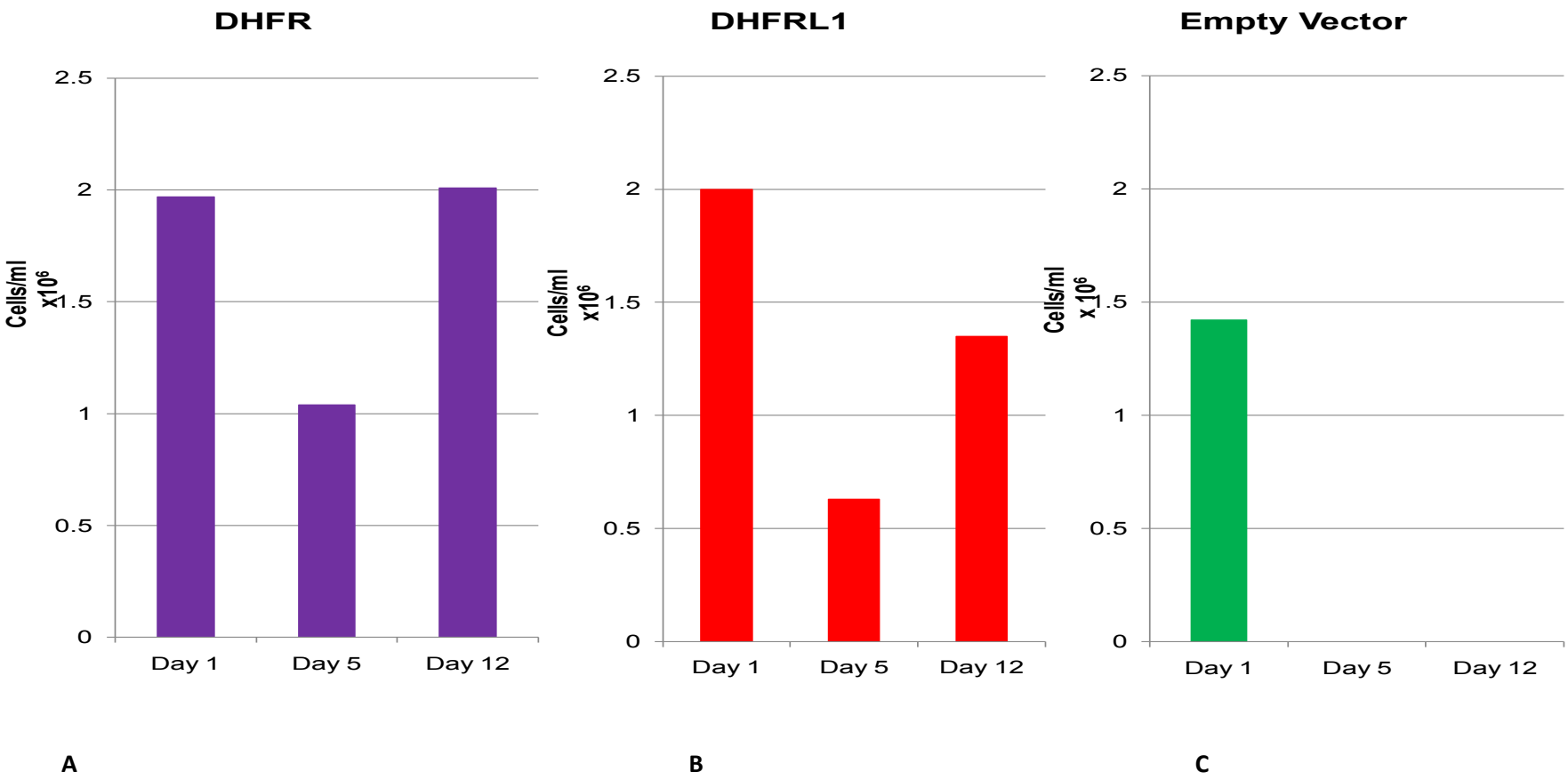
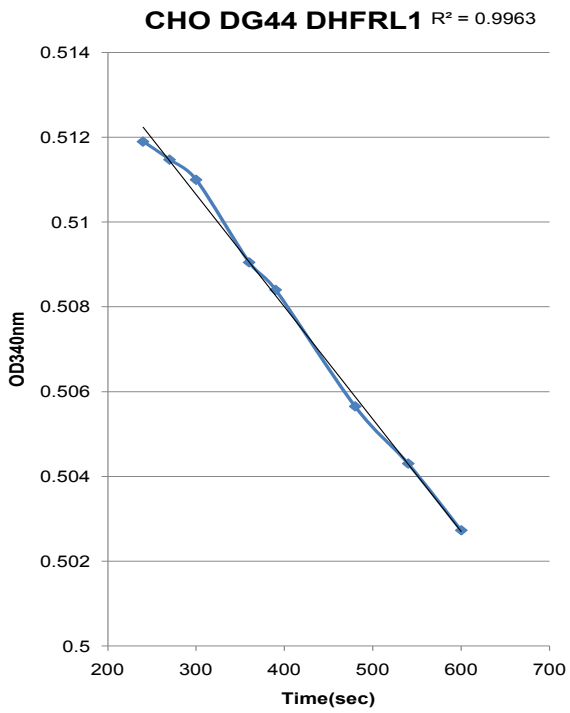
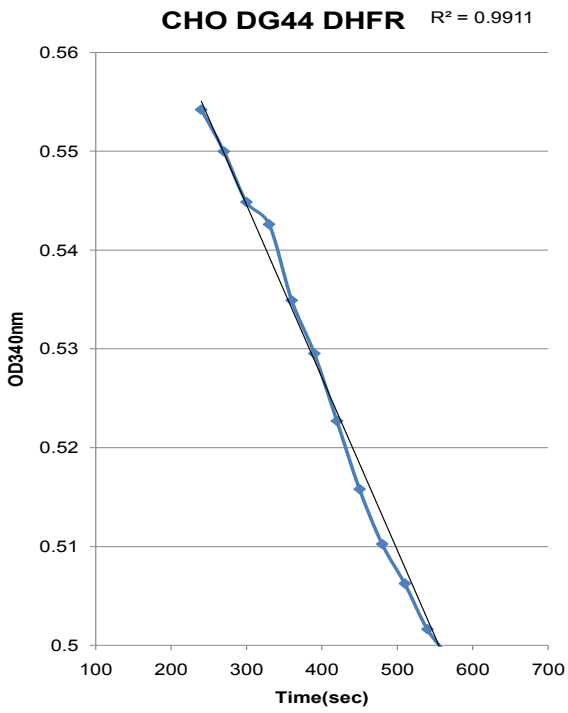


Figure 3.10 Complementation of DHFR negative phenotype in a mammalian system. Cell Counts of transfected CHO DG44 cells after switching cells to media without supplements. Cells were counted after 1, 5 and 12 days in complementation media. Cells transfected with either DHFR **(A)** or DHFRL1 **(B)** had some cell death on day 5 however by day 12 both sets of cells had recovered and were growing well in the complementation media. The cells transfected with DHFR grew more quickly than those transfected with DHFRL1. (**C**) Cells transfected with the empty vector only did not survive without supplement.





A

Specific Activity = 0.0207µmol/min/mg

B

Specific Activity = 0.1326µmol/min/mg

Figure 3.11 Measurement of DHFR enzyme activity in transfected CHO DG44 DHFR negative cells. Protein was harvested from transfected CHO DG44 cells after 12 days in complementation media and enzyme activity was measured. Enzyme activity is equivalent to a drop in absorbance at A_{340nm} over a 10 minute period. **(A)** DHFR activity for CHO DG44 cells transfected with recombinant DHFRL1. Specific activity= 0.0207µmol/min/mg protein. **(B)** DHFR activity for CHO DG44 cells transfected with recombinant DHFR. Specific activity = 0.1326µmol/min/mg protein.

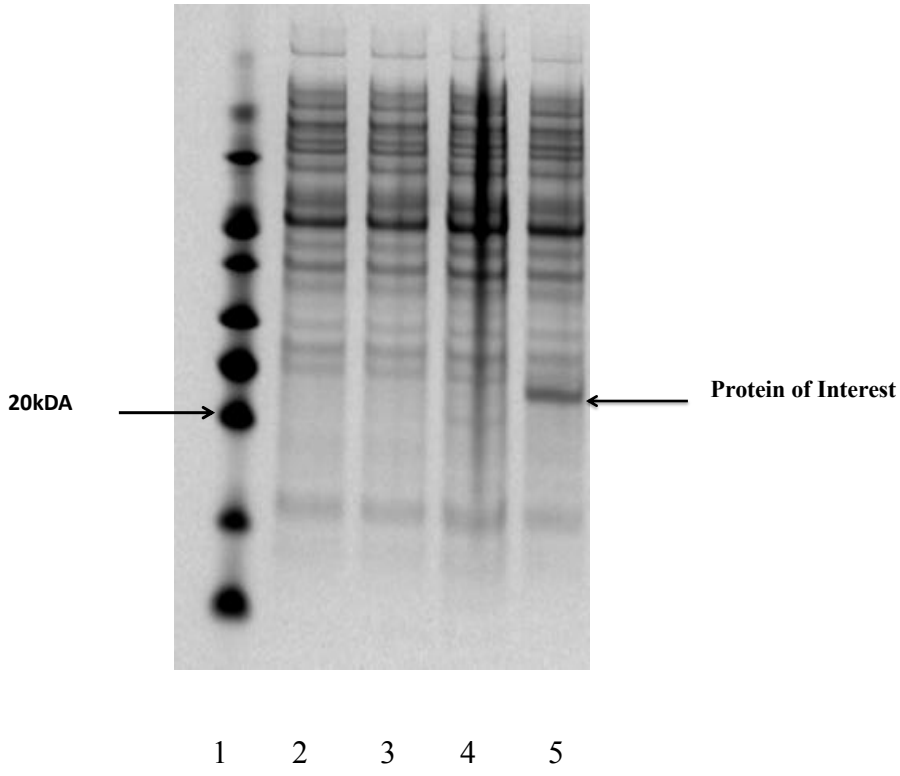


Figure 3.12 SDS-PAGE analysis of BL21-A1 soluble + in soluble fraction (HIS Tag). Protein was harvest from both induced and un-induced samples and separated into soluble and un-soluble fractions after incubation at 37^{O}C overnight. **Lane 1** molecular marker, **lane 2** un-induced soluble fraction, **lane 3** induced soluble fraction, **lane 4** un-induced insoluble fraction and **lane 5** induced insoluble fraction.

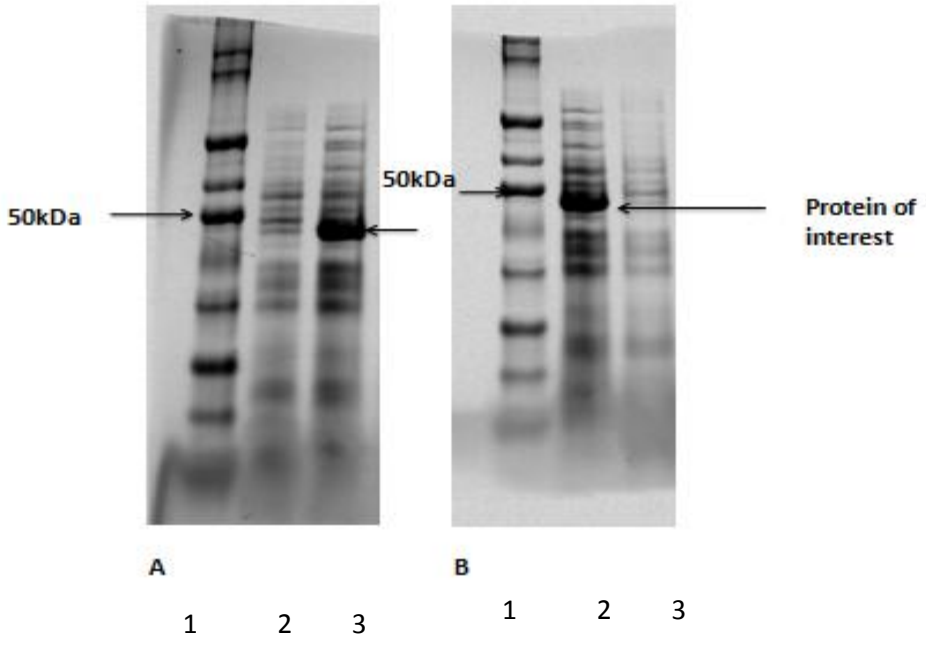


Figure 3.13 SDS-PAGE analysis of BL21-A1 soluble fraction (GST Tag). Protein was harvest from both induced and un-induced samples and separated into soluble and un-soluble fractions after incubation at 37^{O}C overnight. **(A)** Bl21-A1 transformed with DHFRL1. Lane 1 shows molecular marker, Lane 2 un-induced sample and Lane 3 induced sample. **(B)** Bl21-A1 transformed with DHFR. Lane 1 shows molecular marker, Lane 2 induced sample and Lane 3 un-induced sample. The protein of interest is clearly present in the induced samples and not the un-induced.

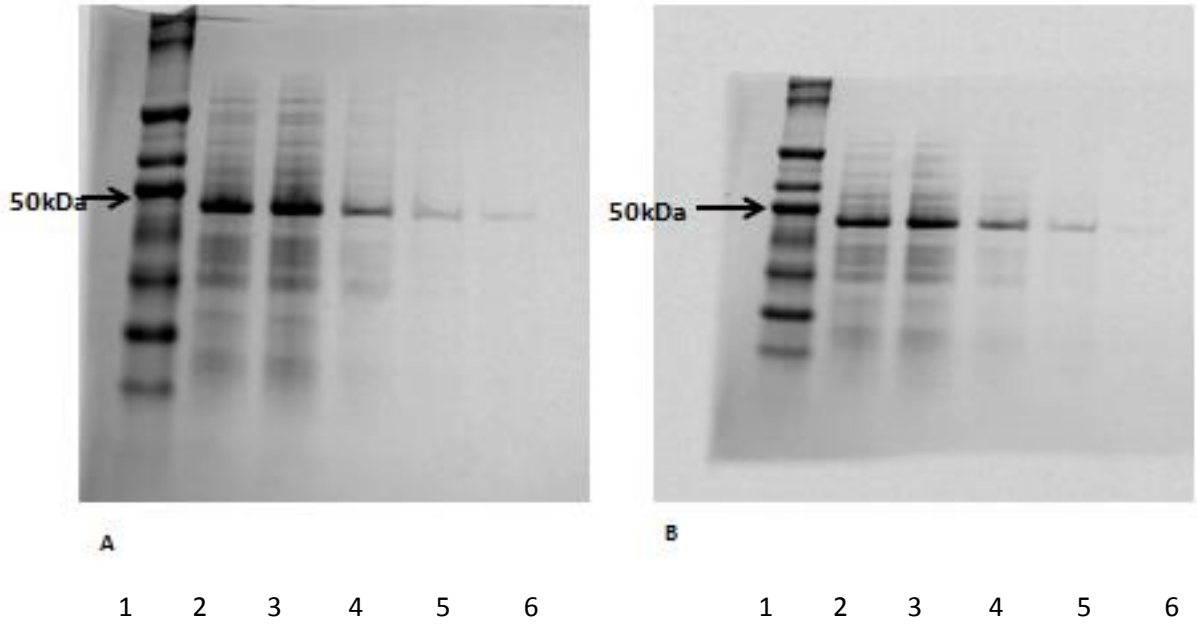


Figure 3.14 SDS-PAGE analysis of protein purification fraction. The GST tagged protein was purified by binding the GST tag to glutathione agarose. The purified protein was eluted using 35mM glutathione. **(A)** GST-DHFRL1 purification. Lane 1 molecular marker, lane 2 flow-through, lane 3 wash step, lane 4 elute 1, lane 5 elute 2 and lane 6 elute 3. **(B)** GST-DHFR purification. Lane 1 molecular marker, lane 2 flow-through, lane 3 wash step, lane 4 elute 1, lane 5 elute 2 and lane 6 elute 3.

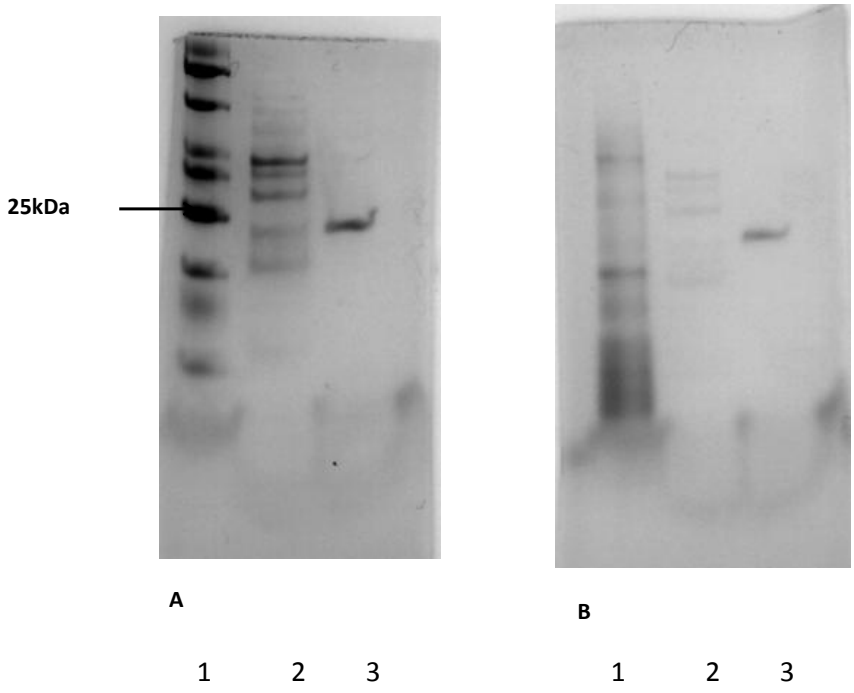


Figure 3.15 SDS-PAGE analysis of TEV cleavage of GST tag. The GST tagged protein was treated with TEV enzyme and incubated overnight at 30^{o}C. The sample was then passed through the glutathione agarose column once more and the purified protein was released in the wash step while the GST remained bound to the column. **(A)** DHFRL1 protein samples. Lane 1 molecular marker, lane 2 flow through and lane 3 purified protein. **(B)** DHFR protein samples. Lane 1 molecular marker, lane 2 flow through and lane 3 purified protein.

Table 3.1 Kinetic analysis of recombinant DHFRL1 *versus* DHFR

Enzyme	Dihydrofolic Acid*		NADPH†				
	K_m‡	V_{max}	K_m¶	V_{max}	Specific Activity	K_{cat} ¥	K_{cat}/K_m
	µM	µmol/min/ml	µM	µmol/min/ml	µmol/min/mg	sec^{-1}	$sec^{-1}\mu M^{-1}$
DHFR							
0.2 – 2.0 µM‡	0.28 ± 0.08§	0.00135 ± 0.0001	3.6 ± 0.4	0.021 ± 0.0004	6.1 ± 0.3	17.4	62.14
DHFRL1							
0.2 - 2.0 µM‡	0.92 ± 0.08	0.0023 ± 0.0001	3.4 ± 0.7	0.038 ± 0.001	4.3 ± 0.3	30	32.61

*At constant 50 µM NADPH

† At constant 60 µM Dihydrofolic acid

‡ Dihydrofolic acid concentration ranges

§ Standard Error

¶ NADPH concentration ranged from 2.5-150 µM

¥ $K_{cat} = V_{max} / [E]_t$

Chapter 4

The role of DHFRL1 in regulating both itself and DHFR

4.1 Introduction

The control of gene expression is essential for the maintance of a healthy cell. Numerous diseases, including cancer, often show alternative gene expression profiles when compared to normal cells. The cell has a number of options to control gene expression both within the nucleus at the transcriptional level and in the cytoplasm at the level of protein translation. The regulation of the translation of mRNA is a common mechanism of control that offers the cell a number of advantages including; a more rapid response to environmental influences,[106] a more energy efficient method to "fine – tune" the levels of a cellular protein,[107] and most importantly the process can be easily reversed usually through the removal of the repressor.[108]

Global translational regulation, such as in the event of infection, can be caused by the phosphorylation or de-phosphorylation of iniation factors. [106] However specific translational control of a single mRNA or family of mRNA is usually mediated by RNA – binding proteins.[109] An increasing number of these RNA – binding proteins have been shown to have the capability of binding to their own mRNA molecules revealing the presence of auto-regulating mechanisms within the cell.

A number of folate metabolism enzymes have been shown to belong to this group of auto-regulatory RNA – binding proteins. These include thymidylate synthase (TS), [110] and serine hydroxymethyltransferase (SHMT).[111] Both of these studies showed that each protein specifically interacted with their respective mRNA only. A third folate enzyme, DHFR, has also been shown to act as an auto-regulatory RNA – binding protein.[112] This initial study also showed that the binding process could be reversed for DHFR not only by the addition of either its substrate dihydrofolate or co-factor NADPH but also the commonly used inhibitor methotrexate. Follow up studies have since identified the specific RNA sequence, between 500 and 653bp, in the coding region where the DHFR protein binds.[113] Other studies had previously shown that RNA binding proteins generally targeted stem and loop structures as they allow single stranded regions to be exposed for binding.[114] The nucleotides between positions 500 and 653 revealed the presence of two loop structures, one at position 559-585 and the other at 590 and 621, which would be suitable for DHFR protein binding, see Figure 4.1.[114] This regulatory mechanism has also been explored from the protein avenue with a number of the essential amino acids required for RNA recognition identified.[115] Cys6, Ile7, Arg28 and Phe34 have all been identified as

critical residues as mutations at these positions significantly reduce the ability of the protein to bind RNA. Furthermore the study also revealed that the dual functions of RNA binding and enzyme activity are not carried out by the same domains within the protein.

With the discovery of a second dihydrofolate reductase, namely DHFRL1, this chapter explores whether DHFRL1 has a role in this mechanism. The amino acids identified as important for RNA binding are all conserved with DHFRL1. See Figure 3.1 in Chapter 3 for sequence alignment. At the RNA level DHFRL1 is also predicted to have at least two stem structures between 500 and 653bp exposing single RNA strand to protein binding, see Figure 4.2. As DHFR and DHFRL1 have such a high sequence homology it is possible that DHFRL1 acting as an RNA – binding protein could regulate the translation of not only its own mRNA but also that of DHFR. The significance of this mechanism would be particularly relevant to the response of MTX treatment as this inhibitor is already prove to act as an "off" switch for this mechanism. The aim of this chapter is to determine if DHFRL1 can act as an RNA binding protein and if so does the binding of DHFRL1 protein to mRNA inhibits translation?

RESULTS

4.2 RNA Binding Ability

The interactions of DHFRL1 protein and DHFR/DHFRL1 mRNA were examined by RNA electrophoretic mobility shift assay (REMSA). The principle behind this assay is if protein is bound to RNA then a band shift should be visible on the gel. The experimental workflow is shown in Figure 4.3. This method was also used to determine the interaction of DHFR protein with both DHFR and DHFRL1 RNA. This assay was carried out as described in section 2.5. RNA probes for both DHFR and DHFRL1 were constructed from PCR products, amplified using T7 forward primer and DHFR/DHFRL1 reverse primer, which used plasmid DNA from pDEST 15 DHFR/DHFRL1 clones as template. This PCR product (2µg) was then *in vitro* transcribed into RNA using HiScribe ™ T1 In Vitro Transcription Kit and analyzed on a 1% (wt/vol) agarose gel, Figure 4.4. The RNA probes were then 3' end biotinylated using Pierce ® RNA 3' Biotinylation Kit and their efficiency determined by dot blot, Figure 4.5. Three separate reactions were set up;

1. Labelled RNA only
2. Labelled RNA + Protein
3. Labelled RNA + Protein + Unlabelled RNA (the unlabelled RNA acts as a specific inhibitor)

If the protein has bound to the RNA a band shift should be clearly visible in lane 2 only. The results for the binding experiment for DHFRL1 protein are shown in Figure 4.6. A clear band shift is observed when the protein was incubated with not only its own mRNA but also for DHFR RNA clearly indicating that DHFRL1 protein can bind both sets of RNA.

The binding experiments for DHFR protein are shown in Figure 4.7. As it has been previously established by Chu *et al* [106] that DHFR binds its own mRNA this acted as the positive control for these experiments. The DHFR binding experiments also revealed that DHFR protein can bind to DHFRL1 mRNA.

From these experiments it can be concluded that DHFRL1 can act as an RNA binding protein not only for its own mRNA but also for DHFR mRNA and in turn DHFR protein also has the ability to bind both its own mRNA and that of DHFRL1.

4.3 Translational Inhibition

Having established through a series of REMSA experiments that DHFRL1 protein could act as an RNA binding protein for both its own mRNA and that of DHFR the next step was to determine if this binding led to translational inhibition. This was tested using a rabbit reticulocyte lysate *in vitro* translation system as outlined in section 2.6. An overview of the experiment is outlined in Figure 4.8.

RNA was transcribed as outlined in section 2.5.1 using linerized plasmid DNA from pDEST15 GST DHFR/DHFRL1 clones. After *in vitro* transcription the RNA was analyzed on a 1% agarose gel, see Figure 4.9. The translation reactions were set up as follows:

1. RNA only
2. RNA + DHFR protein
3. RNA + DHFRL1 protein
4. RNA + GST protein (this acted as a negative control as GST does not bind to either DHFR or DHFRL1 RNA)

As the REMSA experiments showed that DHFRL1 could bind both its own mRNA and DHFR mRNA *in vitro* translation assays were carried out using both sets of RNA bound to DHFRL1 protein. The results shown in Figure 4.10 clearly show translational inhibition has occurred where DHFRL1 is bound to either its own mRNA or DHFR mRNA. Similarly Figure 4.11 shows the *in vitro* translational assays carried out for DHFR protein bound to DHFR mRNA, positive control, and DHFRL1 mRNA. Again a clear inhibition of translation was observed in these samples. These results indicate that the binding of both DHFR and DHFRL1 protein to their respective mRNAs subsequently causes the repressed translation of that mRNA. Figure 4.12 shows *in vitro* translational assays carried out using GST only RNA template. This acted as a negative control for the overall experiment and ensured that the results observed for DHFR and DHFRL1 RNA were not due to the GST tag. In Figure 4.12 translation has clearly occurred for all samples including those with DHFR and DHFRL1 protein present.

4.4 Discussion

The interactions of RNA and protein are relevant to almost every step of gene expression from transport to the cytoplasm, to the translational process itself and importantly to the post-transcriptional regulation of the mRNA molecule. Post-transcriptional regulation is a convenient, energy efficient method for allowing the cell to control the levels of certain proteins.

Many RNA-binding proteins target specific mRNA molecules and as a result may only control the translation of one or more proteins. The RNA-binding proteins may have other functions within the cell and so do not necessarily belong to the same protein family. However a general consensus between them is the RNA motif that they bind to. RNA does not exist in the cell as a single stranded molecule rather it forms a more stable secondary structure [116] which results in single strand RNA present in only stem loops or hairpins. These loops are the general target for most RNA binding proteins.[116]

A number of these RNA –binding proteins target their own mRNA molecules in an effective translational auto-regulation mechanism. The protein recognizes and binds to its own mRNA effectively repressing translation. This mode of regulation appears to be common among folate metabolism enzymes with at least three separate enzymes; TS, SHMT and DHFR, operating this mechanism of auto-regulation. These enzymes are also all involved in the *de novo* thymidylate synthesis pathway in both the cytoplasm and the nucleus. A fourth folate enzyme can now be added to this list in the form of DHFRL1 which is also involved in *de novo* thymidylate synthesis in the mitochondria.

Like its sister enzyme DHFR, DHFRL1 has the ability to recognise and bind to its own mRNA molecules resulting in the inhibition of translation. Interestingly DHFRL1 also has the ability to recognise and bind DHFR mRNA molecules; with DHFR also possessing the ability to recognise and bind DHFRL1 mRNA molecules. The capability of both proteins to recognise and bind to each other's mRNA is perhaps unsurprising given the high sequence similarities between the two proteins. At the protein level the amino acids required by DHFR to bind its mRNA are all conserved within DHFRL1 protein. At the RNA level the region of RNA most likely to be bound by protein, 500-653bp, is also highly similar between DHFR and DHFRL1 (see Figure 4.13 for

sequence alignment of this region), with both RNA secondary structures displaying stem loops at this region exposing a single stranded area for either protein to bind.

What are the functional consequences of the cross binding of DHFR and DHFRL1 to each other's mRNA transcripts? As discussed in more detail in Chapter 5, DHFRL1 protein has a strong presence in the mitochondria so therefore only low levels of the protein may reside in the cytoplasm. It is possible that the role DHFRL1 plays in regulating DHFR translation may be related to the cell cycle. At certain stages of the cell cycle, particularly S phase where DHFR translocates to the nucleus for DNA synthesis, larger quantities of DHFR protein are required by the cell however when the cell is in a resting phase it does not require as much DHFR protein so it is possible that DHFRL1 protein works in tandem with DHFR protein to repress the translation of DHFR mRNA molecules. When the cell again requires more DHFR protein to be made, DHFRL1 can release the mRNA molecules bound to it.

Due to the localisation of DHFRL1 to the mitochondria it is possible that DHFR protein binding to DHFRL1 mRNA may be the more physiologically relevant simply because there may be more DHFR protein found in the cytoplasm, and therefore it may play the leading role in regulating the translation of both proteins. As with any protein the cell requires different concentrations at different stages which it must be able to produce in order to survive. The same is most likely true for DHFRL1. DHFRL1 is a protein coded for by a gene in the nucleus, translated in the cytoplasm and then transported to the mitochondria a process which takes time to complete. If a sudden increase in DHFRL1 protein is required by the mitochondria it may be more efficient to have a stock of DHFRL1 transcripts already present in the cytoplasm waiting to be translated but were prevented from doing so by the binding of DHFR protein. While these transcripts are being translated and transported to the mitochondria, the cell has more time to activate transcription of the *DHFRL1* gene and produce more DHFRL1 transcripts which will eventually lead to an increase in DHFRL1 protein. As with the regulation of DHFR protein when the DHFRL1 protein is no longer required by the cell whatever transcripts are left can be prevented from progressing through to translation by the binding of either DHFR or DHFRL1 protein until they are required by the cell again.

The more clinical relevance of this translational regulating mechanism may be in the effect methotrexate (MTX) has on DHFRL1 binding. Previous studies have shown that MTX disrupts

DHFR protein-RNA binding releasing the RNA for translation and thereby increasing levels of DHFR enzyme activity as an initial response to MTX. The effect MTX has on DHFRL1 binding both to its own mRNA and DHFR mRNA is discussed in more detail in Chapter 6.

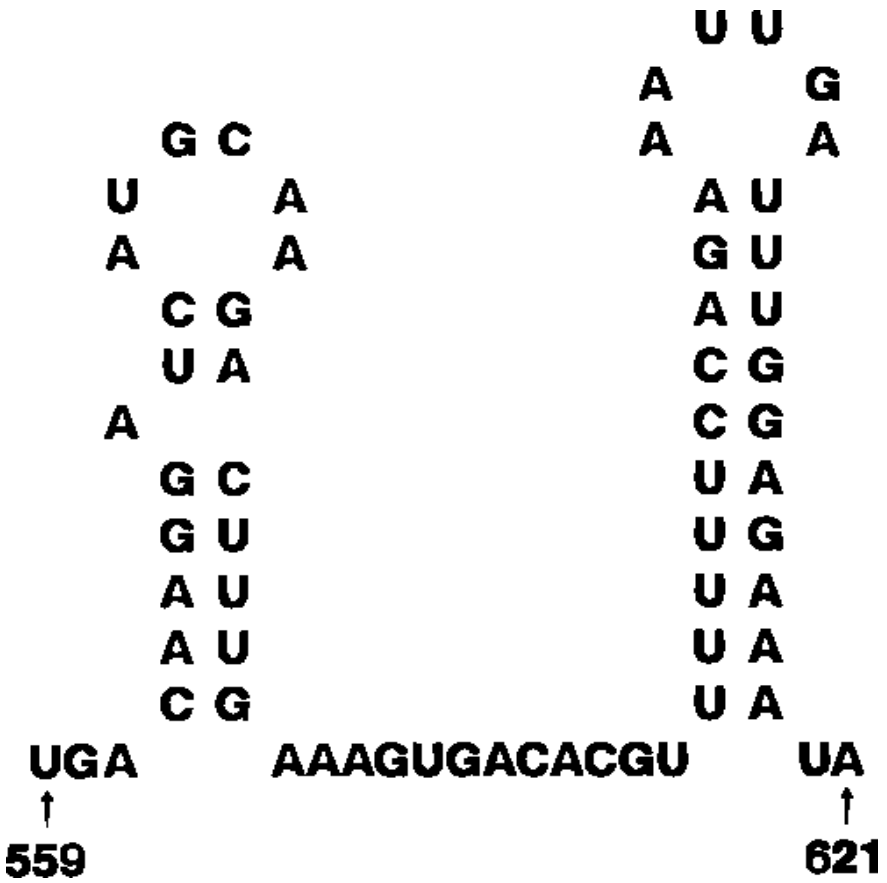


Figure 4.1 Computer generated secondary structure of DHFR mRNA. Image taken from Ercikan-Abali *et al.* (1997). *Biochemistry.* **36:**12314-12322. Nucleotides 500-653bp on the mRNA molecule is where DHFR protein binds to DHFR mRNA. This region contains two loop structures which expose single stranded RNA allowing the protein access to the RNA molecule.

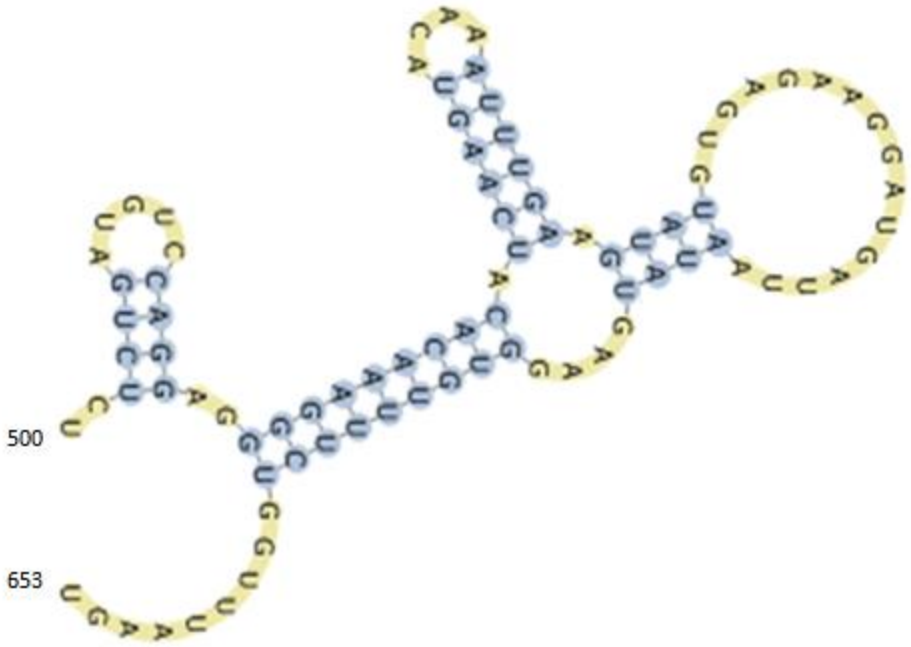


Figure 4.2 Predicted secondary structure of DHFRL1 between bases 500 and 653. The RNA secondary structure prediction software Hotknots [117] (available at www.rnasoft.ca) was used to predict the secondary structure of DHFRL1 RNA, the structure was then visualized using Pseudoviewer. [118] DHFRL1 RNA is predicted to have at least two stem loop structures between bases 500 and 653 exposing single stranded RNA allowing a protein to bind to it. Tai et al [90] identified this region of DHFR RNA as the position of DHFR protein binding with the two stem loop structures present the most likely region to be targeted by the protein.

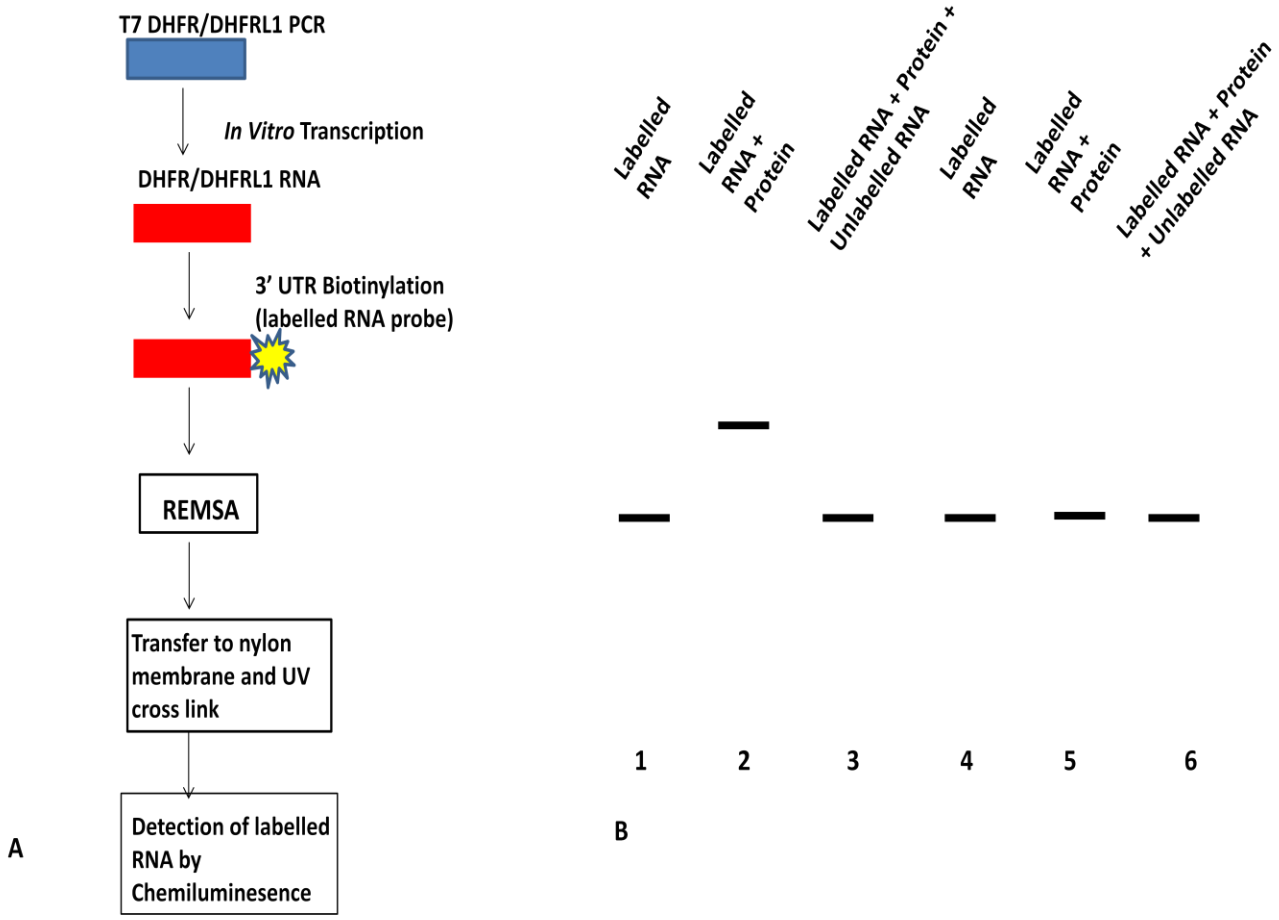


Figure 4.3 Principle of REMSA. (A) DHFR/DHFRL1 RNA was *in vitro* transcribed from PCR product containing T7 promoter. The RNA was then labelled by Biotinylation of the 3'UTR and used with DHFR/DHFRL1 protein in REMSA experiments and run on a non-denaturing SDS gel and then transferred to a nylon membrane. Samples were fixed onto membrane by UV cross – linking and labelled RNA was detected using Chemiluminescence. **(B)** Lanes 1-3 show what the gel should look like if the protein is bound to the RNA. Lane 1 is labelled RNA only, Lane 2 is Labelled RNA + protein, if the protein is bound to the RNA one can see a band shift in this lane compared to lane 1, Lane 3 is labelled RNA + protein + unlabelled RNA, the unlabeled RNA acts as a specific inhibitor and binds to the protein instead of the labelled RNA as a result the labelled RNA is free and the band in this lane is in the same region as lane 1. Lanes 4-6 depict what the gel would look like if the protein is not bound to the RNA. All the bands are at the same region and there is no band shift observed.

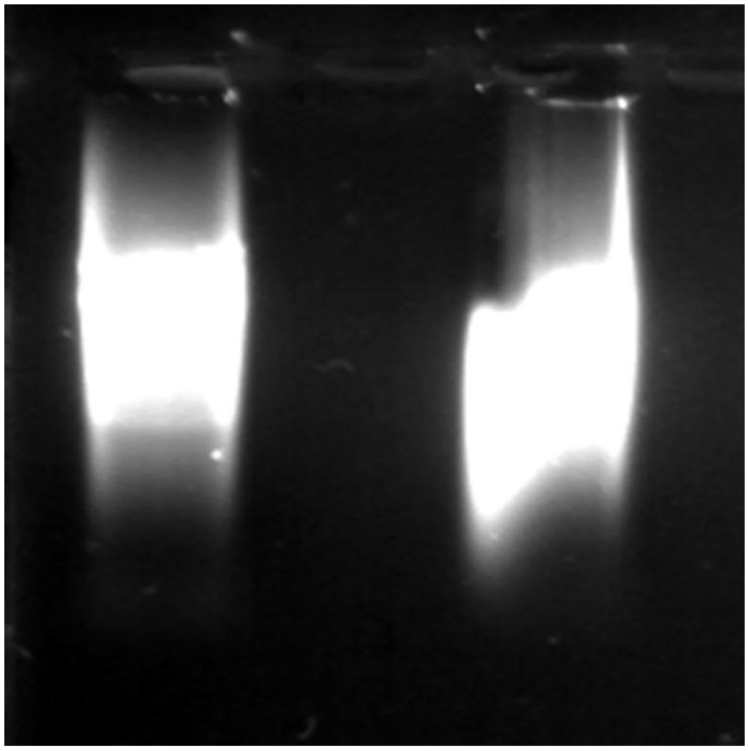


Figure 4.4 In vitro transcribed RNA. RNA was in vitro transcribed using PCR product amplified from pDest15 DHFR/DHFRL1 plasmid DNA. RNA was analyzed on a 1% agarose gel to ensure transcription had occurred and RNA was not degraded. Lane 1 shows *in vitro* transcribed DHFR RNA, lane 2 shows *in vitro* transcribed DHFRL1 RNA.

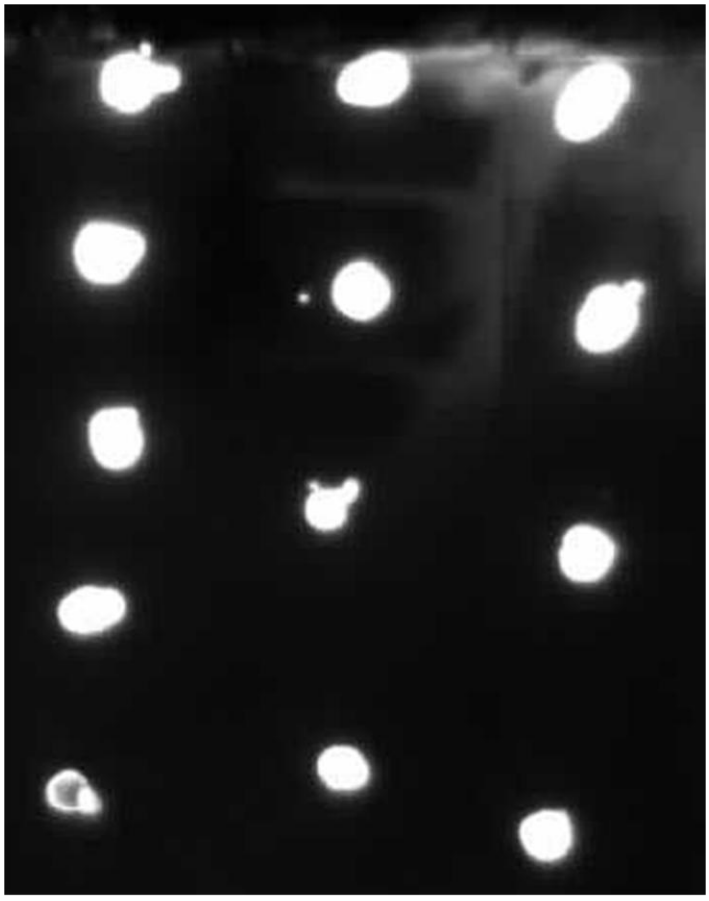
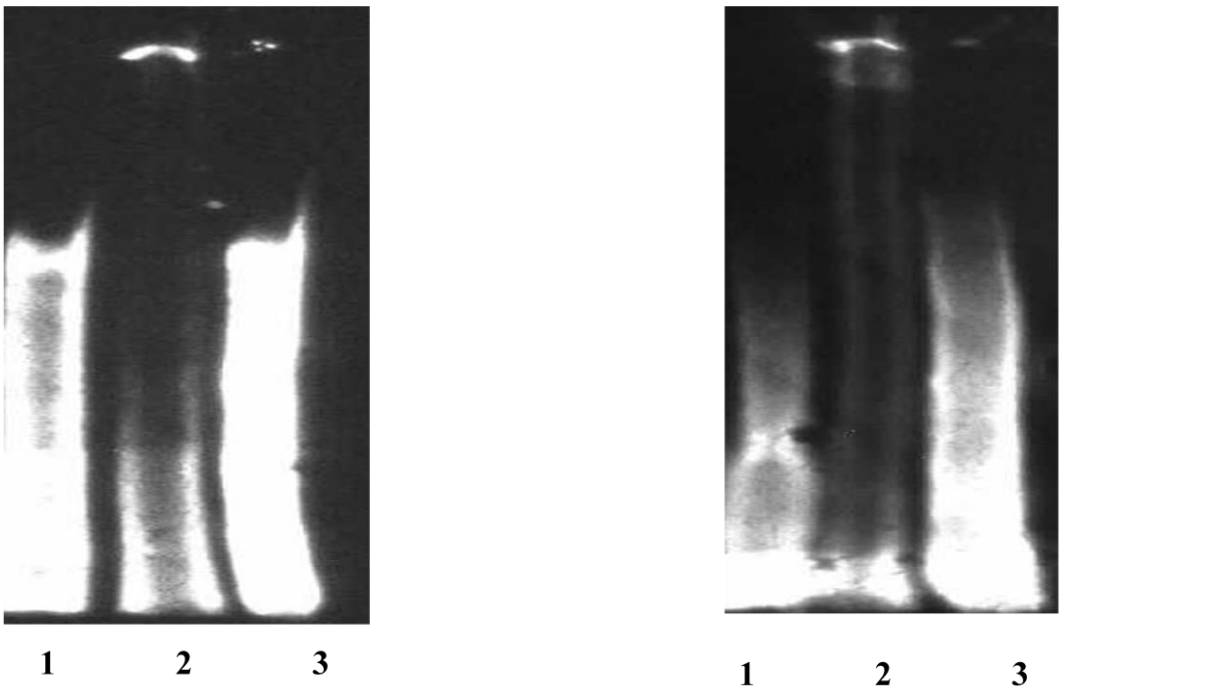


Figure 4.5 Dot Blot to determine efficiency of 3'end Biotinylation of RNA. After *in vitro* transcription RNA was then 3'end Biotinylated. A dot blot was carried out to ensure that RNA was biotinylated. Lane 1 control RNA from kit, lane 2 *in vitro* transcribed DHFR RNA and lane 3 *in vitro* transcribed DHFRL1 RNA.



(A) REMSA DHFRL1 RNA + DHFRL1 protein **(B)** REMSA DHFR RNA + DHFRL1 protein

Figure 4.6 REMSA for DHFRL1 protein bound to either DHFR or DHFRL1 RNA. DHFRL1 protein was incubated with both DHFR and DHFRL1 RNA to determine if it could bind to either RNA. A clear band shift, which indicates binding of protein to RNA, was observed for both DHFR and DHFRL1 RNA indicating that DHFRL1 protein can bind to both RNA.

(A) Lane 1 DHFRL1 labelled RNA only, lane 2 labelled DHFRL1 RNA + DHFRL1 protein and lane 3 labelled DHFRL1 RNA + DHFRL1 protein + unlabelled DHFRL1 RNA.

(B) Lane 1 DHFR labelled RNA only, lane 2 labelled DHFR RNA + DHFRL1 protein and lane 3 labelled DHFR RNA + DHFRL1 protein + unlabelled DHFR RNA

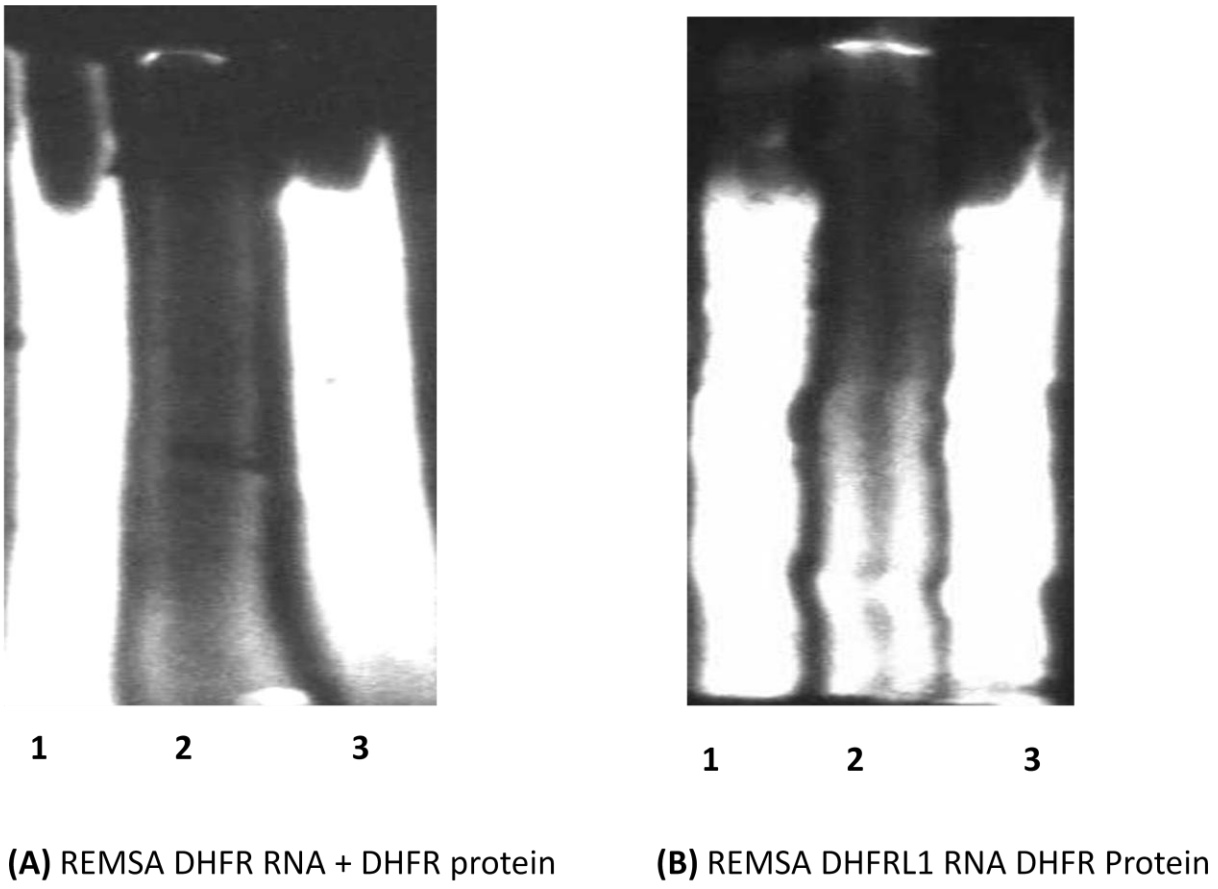


Figure 4.7 REMSA for DHFR protein bound to either DHFR or DHFRL1 RNA. DHFR protein was incubated with both DHFR and DHFRL1 RNA to determine if it could bind to either RNA. A clear band shift was observed for both DHFR and DHFRL1 RNA indicating that DHFR protein can bind to both RNA. The binding of DHFR protein to DHFR RNA acted as a positive control for the overall experiment.

(A) Lane 1 DHFR labelled RNA only, lane 2 labelled DHFR RNA + DHFR protein and lane 3 labelled DHFR RNA + DHFR protein + unlabelled DHFR RNA.

(B) Lane 1 DHFRL1 labelled RNA only, lane 2 labelled DHFRL1 RNA + DHFR protein and lane 3 labelled DHFRL1 RNA + DHFR protein + unlabelled DHFRL1 RNA.

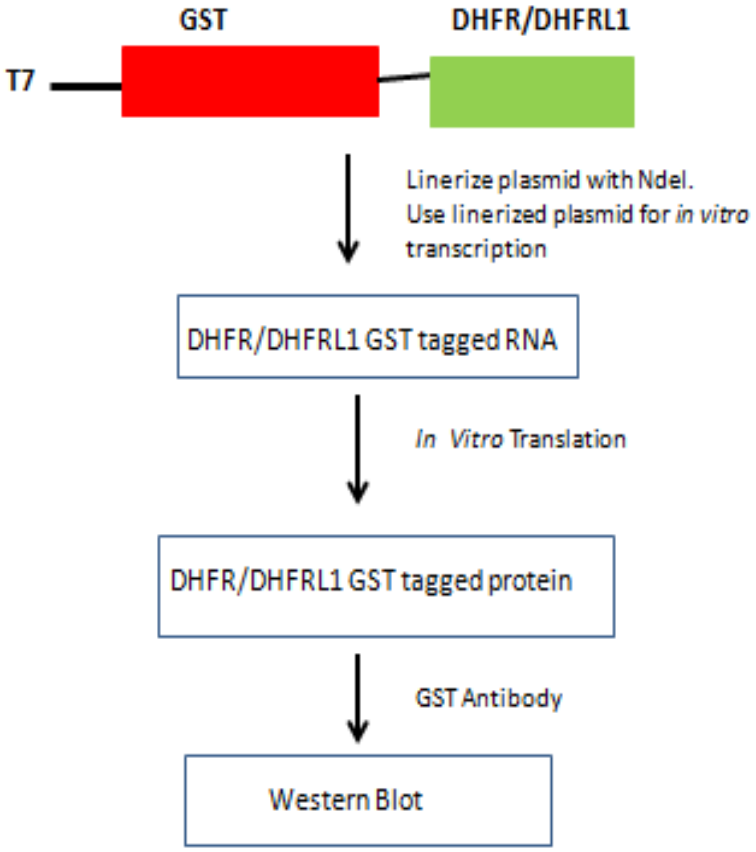
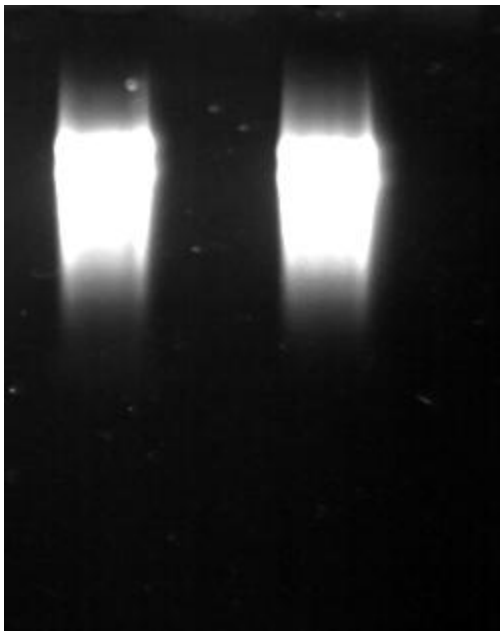


Figure 4.8 Overview of *in vitro* translation experiment. DHFR/DHFRL1 GST tagged RNA was transcribed from linerized DHFR/DHFRL1 GST plasmid DNA. This RNA was then used in the *in vitro* translation assay and translated into GST tagged protein. A GST primary antibody was used in a western blot to detect translated protein.



1 **2**

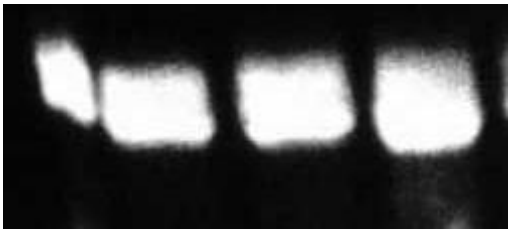
Figure 4.9 **In vitro transcribed RNA.** RNA was analyzed on a 1% agarose gel to ensure transcription had occurred and RNA was not degraded. Lane 1 shows *in vitro* transcribed DHFR GST tagged RNA, lane 2 shows *in vitro* transcribed DHFRL1 GST tagged RNA.

1 2 3 4

Figure 4.10 DHFRL1 *in vitro* translation assay. Translation assay was carried out using DHFRL1 RNA only. Lane 1 DHFRL1 RNA only, Lane 2 DHFRL1 RNA + GST protein, lane 3 DHFRL1 RNA + DHFRL1 protein and lane 4 DHFRL1 RNA + DHFR protein. Bands present in lanes 1 + 2 indicate that translation has occurred however when DHFRL1 RNA is incubated with either DHFRL1 protein (lane 3) or DHFR protein (lane 4) translation is inhibited.

1 2 3 4

Figure 4.11 DHFR *in vitro* translation assay. Translation assay was carried out using DHFR RNA only. Lane 1 DHFR RNA only, lane 2 DHFR RNA + DHFR protein, lane 3 DHFR RNA + DHFRL1 protein and lane 4 DHFR RNA + GST protein. Bands present in lanes 1 + 4 indicate that translation has occurred however when DHFR RNA is incubated with either DHFR protein (lane 2), which acts as a positive control, or DHFRL1 protein (lane 3) translation is inhibited.



1 2 3 4

Figure 4.12 GST *in vitro* translation assay. Translation assay was carried out using GST RNA only. This acted as a control for the overall experiment to ensure that the results observed for DHFR and DHFRL1 were not due to the GST tag. Lane 1 GST RNA only, lane 2 GST RNA + DHFR protein, lane 3 GST RNA + DHFRL1 protein and lane 4 GST RNA + GST protein. Translation occurred in all samples indicating that the results observed for DHFR and DHFRL1 RNA were not as a result of binding to the GST tag on the RNA.

```
          500
 DHFR<   TCTCTGATGT CCAGGAGGAG AAAGGCATTA AGTACAAATT TGAAGTATAT
DHFRL1<  TCTCTGATGT CCAGGAGGGG AAACACATCA AGTACAAATT TGAAGTATG


DHFR<    GAGAAGAATG  ATTAATATGA AGGTGTTTTC TAGTTTAAGT TGTTCCCCCT
DHFRL1<  GAGAAGGATG  ATTAATATGA AGGTGTTTTC TGGTTTAAGT TGTTCCCCCT



DHFR<   CCCTCTGAAA AAAGTATGTA TTTTTACATT AGAAAAGGT    TTTTTGTTGA
DHFRL1< CCCTCTGAGA AAAGTATGCA TTTTTACATT AGAAAAGGGA   CTTTTGTTGA


            653
DHFR<    CTTT
DHFRL1<  CTTC
```

Figure 4.13 A sequence alignment of region 500-653bp of DHFR and DHFRL1 mRNA. The sequence between 500 and 653bp in DHFR mRNA has been shown to be the region where DHFR protein binds to DHFR mRNA. The region is also highly conserved in DHFRL1 mRNA.

Chapter 5

Sub-cellular localization of DHFRL1

5.1 Introduction

Folate-mediated one carbon metabolism is compartmentalized in the cytoplasm, mitochondria and the nucleus. One-carbon metabolism in the cytoplasm provides the building blocks for purine and thymidylate synthesis as well as the remethylation of homocysteine to methionine. While mitochondria one-carbon metabolism is required for the synthesis of serine, purines and glycine among others, it also provides one-carbon units for metabolism in the cytoplasm. Nuclear folate metabolism centres on *de novo* thymidylate biosynthesis.[119] With three distinct centres of folate metabolism in the cell, cellular localization of folate enzymes is important.

DHFR, in its essential role of providing tetrahydrofolate, is localized primarily to the cytoplasm. However in recent years evidence has emerged to suggest that DHFR is sumoylated by SUMO-1 (small ubiquitin-like modifier-1) and through this process is targeted to the nucleus.[120] DHFR is one of three folate enzyme involved in *de novo* thymidylate synthesis, the other two being serine hydroxymethyltransferase (SHMT) and thymidylate synthase (TS). DHFR has also been shown to translocate from the cytoplasm to the nucleus during apoptosis caused by DNA damaging agents.[121] This study showed that DHFR was not pre-requisite for DNA damage induced apoptosis nor did it prevent the apoptosis so the biological significance of this process is still unclear. What both these studies highlight is that DHFR is not only present in the cytoplasm but also enters the nucleus. No evidence of DHFR has been found in the mitochondria. Anderson et al[120] determined that the sumoylation site for DHFR to enter the nucleus was at position 179, the YKFE motif, with the K residue the target for sumoylation modification. This motif is conserved within the DHFRL1 protein indicating that DHFRL1 may also be present in multiple cellular locations.

Aims/Objectives

1. Generation of a GFP expression clone to determine sub-cellular localisation of DHFRL1
2. Investigate potential sumoylation of DHFRL1 protein by SUMO-1 protein.
3. Confirmation of expression or non-expression of DHFRL1 T1 transcript.
4. Investigate expression levels of *DHFRL1* transcripts during the cell cycle
5. Develop DHFRL1 specific antibody to distinguish between endogenous DHFR and DHFRL1 at protein level.

RESULTS

5.2 Generation of GFP DHFRL1 expression clone

A GFP DHFRL1 expression clone was created using the Gateway system as outlined in Section 2.3.7a, similar to the mammalian expression clones created for the complementation study outlined in Section 3.25. The destination vector pcDNA6.2 was used as it contains both the GFP tag at the C terminus and also the CMV promoter required for expression in mammalian cells. Plasmid DNA was purified from the entry clone, (Figure 5.1) and recombined with pcDNA6.2 vector. The recombination reaction was transformed into DH5α cells and positive clones were confirmed by restriction digest, (Figure 5.2) and Sanger sequencing.

5.3 Sub-cellular localization of DHFRL1

In order to determine cellular localization of DHFRL1 unsynchronized HEK 293 cells were grown at a concentration of 1 x 10^5 cells/ml and transfected twice, (Section 2.2.3), with a GFP-tagged DHFRL1 expressing plasmid (Gateway Expression Vector pcDNA6.2-EmGFP). After 48 hours of transfection the cells were visualized using a Zeiss Axio Observer confocal microscope and the images were analyzed using Zen 2008 software. All confocal images were taken by Dr. Nadia Ben Larbi from Dr. Christine Loscher's research group, Dublin City University. Results are shown in Figure 5.3. The confocal images show a strong presence of DHFRL1 in the mitochondria

5.4 Site directed mutagenesis of DHFRL1

The region of greatest difference between DHFR and DHFRL1 is only three amino acids long at the N terminal of the protein. In order to determine if these amino acids are involved in the localization of DHFRL1 to the mitochondria site directed mutagenesis was carried out, as described in section 2.3.9 on the DHFRL1 GFP expression, clone to mutate these amino acids into the three amino acids found in DHFR. Mutagenesis was confirmed by Sanger sequencing. HEK 293 cells were grown at a concentration of 1 x 10^5 cells/ml and transfected twice with 5µg of mutant DHFRL1 plasmid DNA. After 48 hours after transfection localization of the mutant plasmid was visualized by confocal microscopy. The results depicted in Figure 5.4 show that the

mutant DHFRL1 did not appear to be present in the mitochondria while wild type DHFRL1 shows a strong presence in the mitochondria.

This experiment was again repeated and the mitochondria and cytoplasmic fractions isolated and analyzed by western blot. Again GAPDH was used as an endogenous control for the cytoplasm and PDH was used as the endogenous control for the mitochondria. Once again the western blot results correlate with the confocal images and show a strong band for mutant DHFRL1 in the cytoplasm. The results are shown in Figure 5.5.

5.5 Sumoylation of DHFRL1

DHFRL1 has a conserved sumoylation motif, YKFE, which has previously been shown in DHFR to allow the protein to translocate to the nucleus.[120] In order to determine if the sumoylation motif in DHFRL1 was active an *in vitro* sumoylation assay was carried out, as described in section 2.9, on recombinant DHFRL1 protein. The assay was also carried out on recombinant DHFR protein as a positive control. As a negative control for the reaction a mutant SUMO-1 protein was used, this does not have the ability to sumoylate any proteins. The reactions were analyzed by western blot and probed with both DHFR and SUMO-1 antibodies. The results are shown in Figure 5.6. DHFRL1 protein is roughly 21kDa in size and SUMO-1 protein is approximately 16kDa in size. Therefore if SUMO-1 protein is bound to DHFRL1 protein a band should be observed that is approximately 37kDa in size. The western blots for both DHFR and DHFRL1 show a band at the 40kDa marker for blots probed with both DHFR and SUMO-1 antibody indicating sumoylation has occurred. In the blots probed with the DHFR antibody a band is also observed at the 20kDa marker which is the correct size for protein which is not bound to SUMO-1. These results indicate that the sumoylation motif conserved within DHFRL1 is active and that it is possible that the protein is sumoylated bySUMO-1 *in vivo*.

5.6 Confirmation of expression of *DHFRL1* T1 transcript

There are two transcripts present in the database for DHFRL1, T1 and T2, which produce the same amino acid sequence but differ at the 5'UTR with the T1 transcript having a longer 5' UTR. RT-qPCR analysis carried out by Dr. Kirsty O'Brien confirmed the expression of the T2 transcript (Dr. O'Brien's RT-qPCR results are shown in the Appendix). Primers were designed for the T1 transcript which would distinguish between the two transcripts and confirm the expression or non-expression of the *DHFRL1* T1 transcript. RT-qPCR was carried out on RNA isolated from a number of different cell lines and expression of the T1 transcript was confirmed (Figure 5.7). Expression levels of both transcripts were compared revealing that the T1 transcript has a much lower expression level compared to the T2 transcript in all cell lines tested.

5.7 Expression of *DHFRL1* throughout the cell cycle

Many genes are expressed at different levels at certain stages of the cell cycle depending on their role within the cell. For example DHFR translocates to the nucleus during G_1/S phase to take part in *de novo* thymidylate synthesis which is required for DNA replication, therefore as more of the protein is required expression of the gene increases during G_1 and S phases of the cell cycle. For DHFRL1 there are two RNA transcripts annotated in the database, T1 and T2, both of which encode for the same 187 amino acid protein. The transcripts differ at the 5' UTR with the T1 transcript having a larger 5'UTR than the T2 transcript. Expression of both of these transcripts was analyzed in cell cycle regulated HEK 293 cells by RT-qPCR using primers which were designed to differentiate between the two transcripts.

HEK 293 cells were grown in a T_{75} flask at a concentration of 5×10^5 cells. The cells were cell cycle sorted as described in section 2.2.5. The percentage of cells at each stage of the cell cycle was determined by flow cytometry using a FACSCalibur flow cytometer. Results are shown in Figure 5.8. For cells treated with nocadozole the highest percentage are in G_2 phase, serum starved cells show highest percentage in G_0/G_1, L-mimosine treated cells show highest percentage in G_1/S phase and cells treated with a double thymidine block show the highest percentage in S phase. Once the stage of cell cycle arrest was confirmed in these cells RNA was isolated as described in section 2.3.3 (Figure 5.9) and RT-PCR was performed as outlined in section 2.3.5, to synthesize cDNA. An RT-qPCR reaction was then performed as described in

section 2.3.6. Expression levels of DHFR, DHFRL1 T1 and DHFRL1 T2 were all measured and compared to levels of the endogenous control β-actin. Results are shown in Figure 5.10. As expected expression levels of DHFR are at their highest during S phase of the cell cycle. The DHFRL1 T2 transcript also shows its highest level of expression during the S phase of the cell cycle although expression levels are lower than that of DHFR. The DHFRL1 T1 transcript expression levels are relatively similar throughout the cell cycle and do not appear to increase significantly at any stages of the cell cycle. Statistical analysis using Anova show that the results for both DHFR and DHFRL1 T2 are significant with both having a p value below 0.05. DHFRL1 T1 has a p value above 0.05 indicating that there is no statistically significant change in expression levels of this transcript during the cell cycle. Statistical results are shown in Table 5.1.

5.8 DHFRL1 specific antibody

Due to the high similarity between DHFR and DHFRL1 it is extremely difficult to differentiate between the two at a protein level. Commercially available antibodies to both DHFR and DHFRL1 are likely to cross-react and pick up both proteins. The development of an antibody that would be specific to DHFRL1 and not cross react with DHFR would be a major advantage in studying both these proteins. To that end a peptide was designed against the region of most difference between the two proteins at the N terminal. The peptide was commercially synthesized by Cambridge Bioscience and conjugated to KLH. This peptide was then used to immunize balbc mice as described in section 2.10a. Serum was isolated from mice after 21 days and titre levels were measured by ELISA as described in section 2.10b. Mice were given booster shots of the peptide a total of five times.

Serum titre levels were measured for binding of DHFRL1 and DHFR. Ideally the titre ELISA's should show a clear difference between wells coated with DHFRL1 and those coated with DHFR. If the serum contained a high titre of antibodies specific to DHFRL1 then the DHFRL1 graph should not level out until the high serum dilutions i.e. over 1:100,000 and the DHFR graph should level out at a much lower dilutions. An example of expected results is shown in Figure 5.11.

The actual titre results obtained are shown in Figure 5.12. They clearly show that there is little difference between in antibodies binding to DHFR and DHFRL1. The number of these antibodies is also quite low with both graphs levelling out at the 1:10,000 serum dilutions.

The results were the same with each booster injection given to the mice and after five booster shots with no significant increase in titre levels it was concluded that isolating a DHFRL1 specific antibody from these mice would be extremely difficult and the study did not proceed to the next stage.

5.9 Discussion

The mitochondria are a well established centre of folate metabolism with three distinct process of folate metabolism occurring there; 1) the transfer of one-carbon units to the mitochondrial pool of tetrahydrofolate, 2) the inter-conversion of the activated one carbon unit carried by tetrahydrofolate and 3) the release of one carbon units from tetrahydrofolate to be exported to the cytoplasm.[7]

Tetrahydrofolate is the starting point for all folate reactions in the mitochondria. As DHFR does not localize to the mitochondria[122] it was assumed dihydrofolate could not be reduced to tetrahydrofolate in the mitochondria itself so it had to be imported from the cytoplasm. The localization of DHFRL1 to the mitochondria reveals that some tetrahydrofolate is actually produced in house. In a recent study by Anderson et al [102] DHFRL1 was shown to be involved in *de novo* thymidylate biosynthesis in the mitochondria. Thymidylate synthesis is essential for the replication of both nuclear and mitochondrial DNA and had previously been demonstrated in both the cytoplasm and the nucleus. [123] [124] *De novo* thymidylate synthesis is carried out by thymidylate synthesis (TS), serine hydroxymethyltransferase (SHMT) and DHFR in both the cytoplasm and the nucleus. However Anderson et al conclusively proved, with siRNA technology, that DHFR is replaced by DHFRL1 in the mitochondrial *de novo* thymidylate biosynthesis pathway.

The mechanism of mitochondrial localization is unclear given the lack of any obvious sequence that would indicate DHFRL1 is targeted to the mitochondria. Mitochondria targeting sequences (MTS) do not appear to share a consensus primary sequence rather they share similar overall characteristics. Most MTS lack acidic residues and are rich in the positively charged amino acids

arginine and lysine and the hydroxylated amino acids serine and threonine.[125] A secondary feature common among MTS is the formation of an amphiphilic α helices found on the surface of the protein.[126] Hurt *et al.* found that amino acids 1-85 on mouse dihydrofolate reductase had the potential to be a MTS; however, it was inactive within the folded protein.[127] These amino acids are highly conserved within DHFRL1 and, therefore, the localization of DHFRL1 in the mitochondria may be related to the folding of the enzyme following translation, possibly revealing the presence or absence of amino acids that facilitate its import. The region of greatest difference between DHFR and DHFRL1 amino acid sequences is just three amino acids long at the N terminus. In DHFRL1 these consist of an aromatic amino acid (F) and two of the same hydrophobic amino acid (L), while in DHFR these amino acids consist of a hydrophobic amino acid (V), a small amino acid (G) and a nucleophilic amino acid (S). When these amino acids in DHFRL1 were changed back into the amino acids found in DHFR the mutant DHFRL1 no longer appeared to have a strong presence in the mitochondria indicating that these three amino acids are somehow involved in the localization of DHFRL1 to the mitochondria.. It may be possible that these amino acids change the tertiary structure of the protein which allows the MTS to be on the surface of the protein instead of buried within the protein. It may also be possible that these amino acids are involved in a cell signaling mechanism which signals to another protein to transport DHFRL1 to the mitochondria. However the exact mechanism of how these amino acids are involved remains unclear

A large number of proteins which are required to function in separate organelles of the cell are first translated in the cytoplasm and then transported to their destination organelle by varying methods of post-translational modification. One such method is sumoylation which involves conjugating a small ubiquitin-like modifier (SUMO) protein to a lysine residue of the target protein.[128] This family of proteins consists of SUMO-1, SUMO-2 and SUMO-3 which generally bind to the consensus motif YKXD/E where K is the lysine residue where conjugation occurs.[129] As previously discussed DHFR is sumoylated by SUMO-1 which allows it to be translocated to the nucleus at certain stages of the cell cycle. The sumoylation motif is conserved within DHFRL1 and an *in vitro* assay carried out on recombinant DHFRL1 protein shows that this motif is active. Although this does not prove that DHFRL1 is sumoylated by SUMO-1 protein *in vivo* it does open up the possibility that DHFRL1 can be translocated through post-translational modification. It may be possible that both DHFR and DHFRL1 undergo sumoylation and are

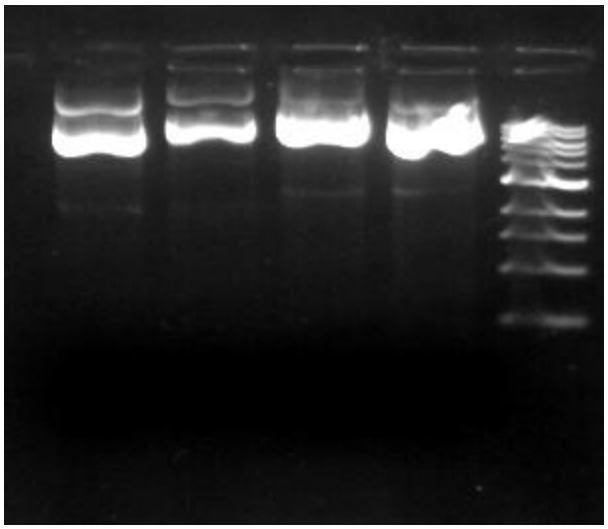
transported to the nucleus. However what role DHFRL1 would have in the nucleus is unclear given that DHFR has already been shown to be involved in *de novo* thymidylate synthesis in the nucleus. Further investigation is required in a cell culture model in order to confirm the presence or absence of DHFRL1 in the nucleus before a hypothesis of its role there can be developed.

DHFRL1 in the mitochondria is involved in *de novo* thymidylate synthesis which is important for mitochondrial DNA replication. Unlike nuclear DNA replication which is regulated in a cell cycle dependent manner, mitochondrial DNA replication appears to be less tightly regulated and can occur at any stage of the cell cycle [130] although a study carried out by Pica-Mattoccia et al[131] found that in HeLa cells mitochondrial DNA replication accelerated in the S phase of the cell cycle. DHFRL1 has two transcripts annotated in the database both of which code for the same protein but differ at the 5'UTR with the T1 transcript having a loner 5'UTR compared to the T2 transcript. In cell cycle synchronized HEK 293 cells, the expression of the T1 transcript remained stable throughout the cell cycle with a slight increase at the S phase, although changes in T1 expression levels were not statistically significant. Similar to the DHFR transcript the T2 transcript showed a 2.5 fold increase in S phase compared to G_0/G_1 phase which would indicate that more DHFRL1 protein is required during S phase. Although it is difficult to determine from RNA data alone why DHFRL1 expression is increased in S phase it is possible that if mitochondrial DNA replication is increased during this phase of the cell cycle when more DHFRL1 protein would be required. However this would need to be investigated at the protein level to determine the exact location of DHFRL1 protein during S phase.

A major challenge in characterizing DHFRL1 at the protein level is being able to distinguish between endogenous DHFR and DHFRL1. Current commercially available antibodies for both DHFR and DHFRL1 have a high probability of binding to both proteins. An attempt to produce a DHFRL1 specific antibody as described in section 5.7 using a synthesized peptide of the region most different between the two proteins proved unsuccessful. Alternative strategies to develop a DHFRL1 specific antibody may be employed with more success. These may include 1) immunizing a different animal such as a rabbit or a different strain of mouse, 2) synthesize alternative peptides or even immunize host animal with whole DHFRL1 protein, 3) an alternative conjugate to (keyhole limpet hemocyanin) KLH such as BSA may also be used. An alternative method to identify endogenous DHFR and DHFRL1 may be mass spectrometry which is already

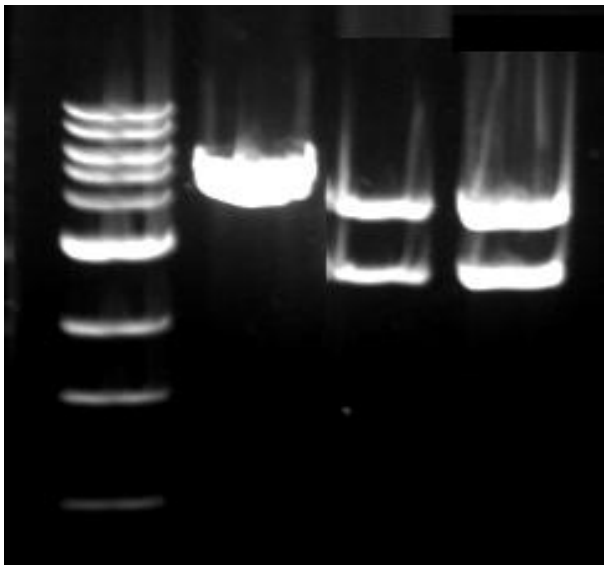
used to identify individual proteins in multi-protein complexes. [132] However a disadvantage with this method is that it requires expert training and specialist equipment.

Although the evidence to date would indicate that DHFRL1 maintains a strong presence in the mitochondria until such a time as endogenous DHFR and DHFRL1 can be identified and studied separately the possibility that DHFRL1 may localize to other cellular compartments such as the cytoplasm or nucleus cannot be ruled out.



1 2 3 4 5

Figure 5.1 Plasmid DNA purified from DHFR/DHFRL1 entry clone. Lane 1+2 DHFR entry clone, **lane 3+4** DHFRL1 entry clone and **lane 5** 1kB ladder.



1 2 3 4

Figure 5.2 Restriction digest of pcDNA 6.2 DHFR/DHFRL1 clones. Plasmid DNA from the DHFR and DHFRL1 clones were treated with *Bgl* II enzyme. The enzyme does not cut the destination vector but should cut both the DHFR and DHFRL1 clones at 2 sites giving 2 bands on the gel. **Lane 1** 1kB ladder, **lane 2** empty vector, **lane 3** DHFR clone, **lane 4** DHFRL1 clone.

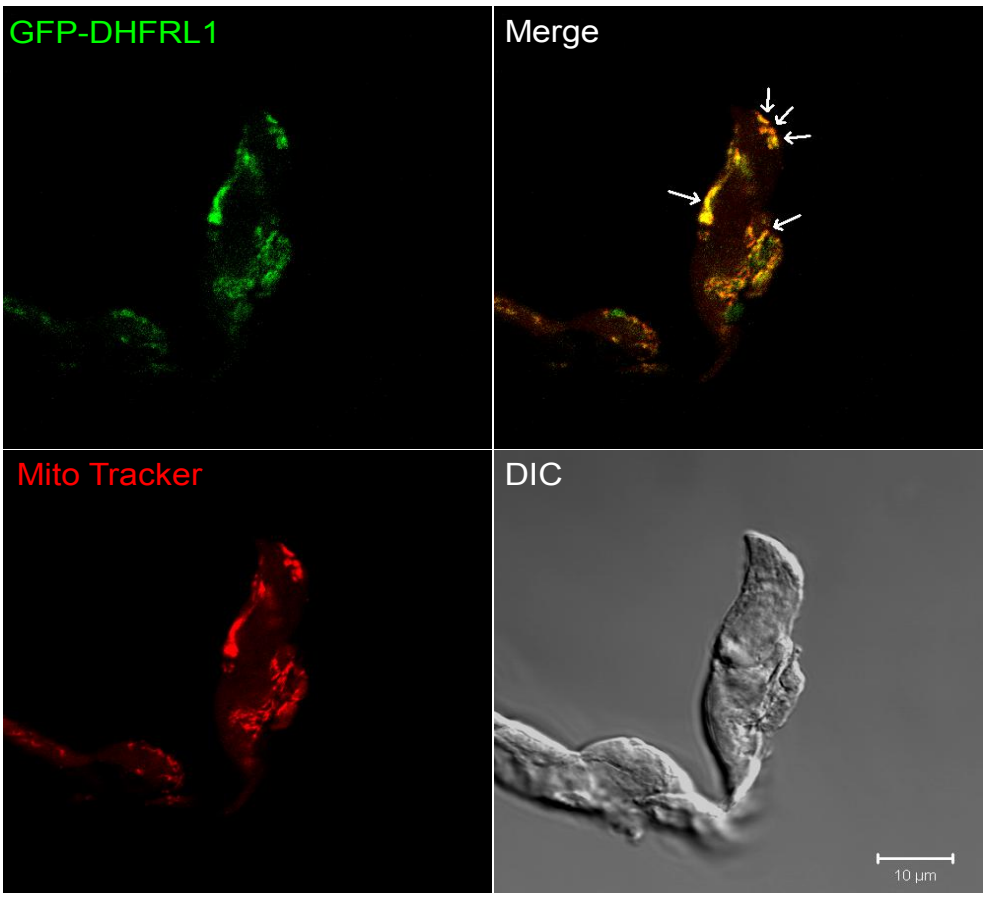


Figure 5.3 Localisation of GFP-DHFRL1 into mitochondria by immunofluorescence. GFP-DHFRL1 (Green) and Mito Tracker CMTMRos (Red, Molecular Probes, Invitrogen) Arrows show localisation of GFP-DHFRL1 into mitochondria. HEK cells were grown on cover slips (1×10^5 cells/ml) and transfected twice with a GFP-tagged DHFRL1 expressing plasmid. After 48 hrs transfection, cells were incubated for 20 mins at 37 C with Mito Tracker at 200 nM. Then cells were fixed in paraformaldehyde on ice for 30 mins. Following rinsing 3×5 min in PBS-baths, the slides were mounted on slides with antifade medium (Dako). Slide preparations were observed using a Zeiss Axio Observer. Z1 equipped with a Zeiss 710 and ConfoCor 3 laser scanning confocal head (Carl Zeiss, Germany). Images were analysed using ZEN 2008 software.

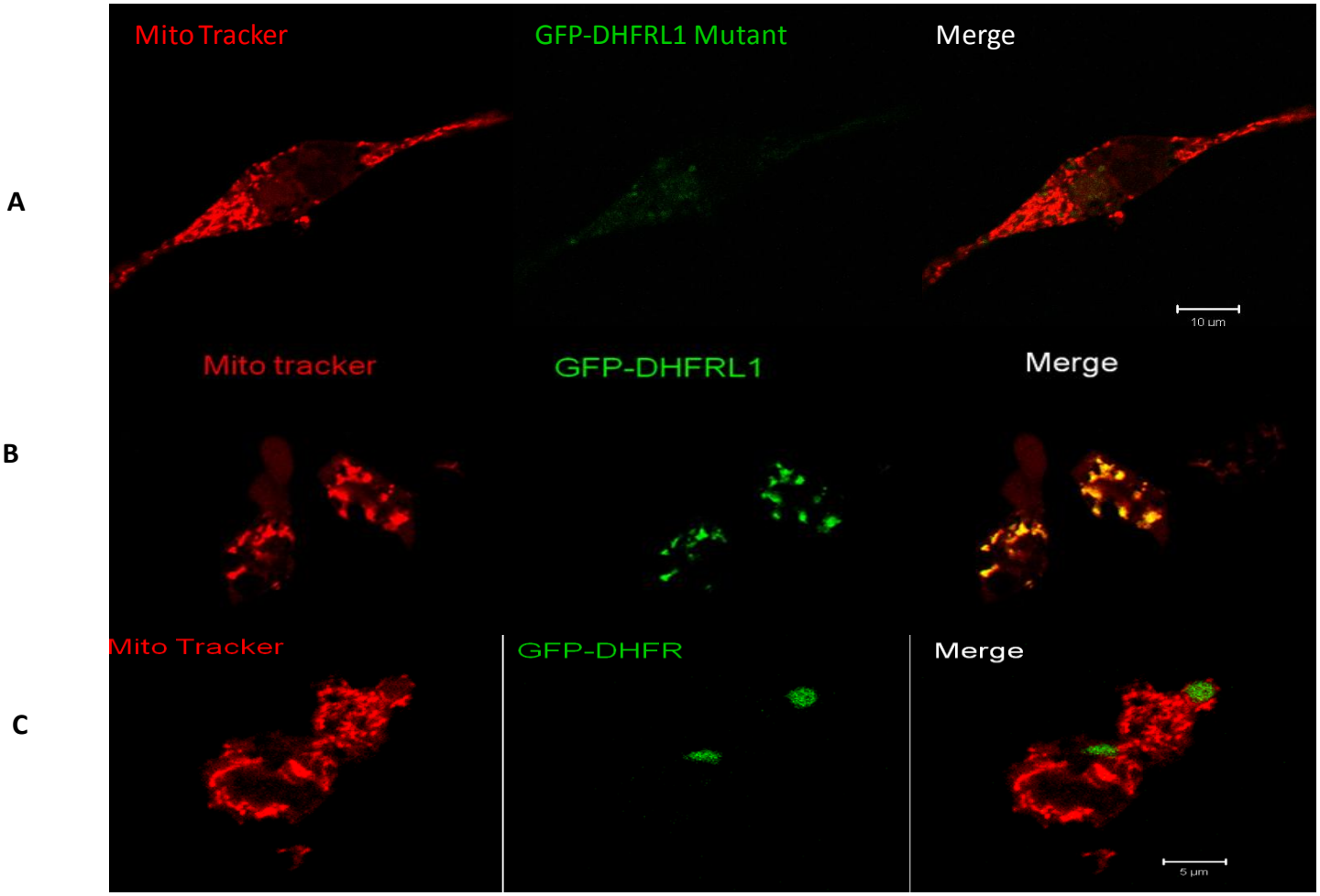


Figure 5.4 Sub-cellular localisation of mutant DHFRL1 by immunofluorescence. . HEK cells were grown on cover slips (1×10^5 cells/ml) and transfected twice with a GFP-tagged mutant DHFRL1 (A), GFP-DHFRL1 (B) or GFP-DHFR. 48 hours later localization was visualised using a confocal microscope. Both DHFR and the mutant DHFRL1 localize to the cytoplasm while DHFRL1 localizes to the mitochondria.

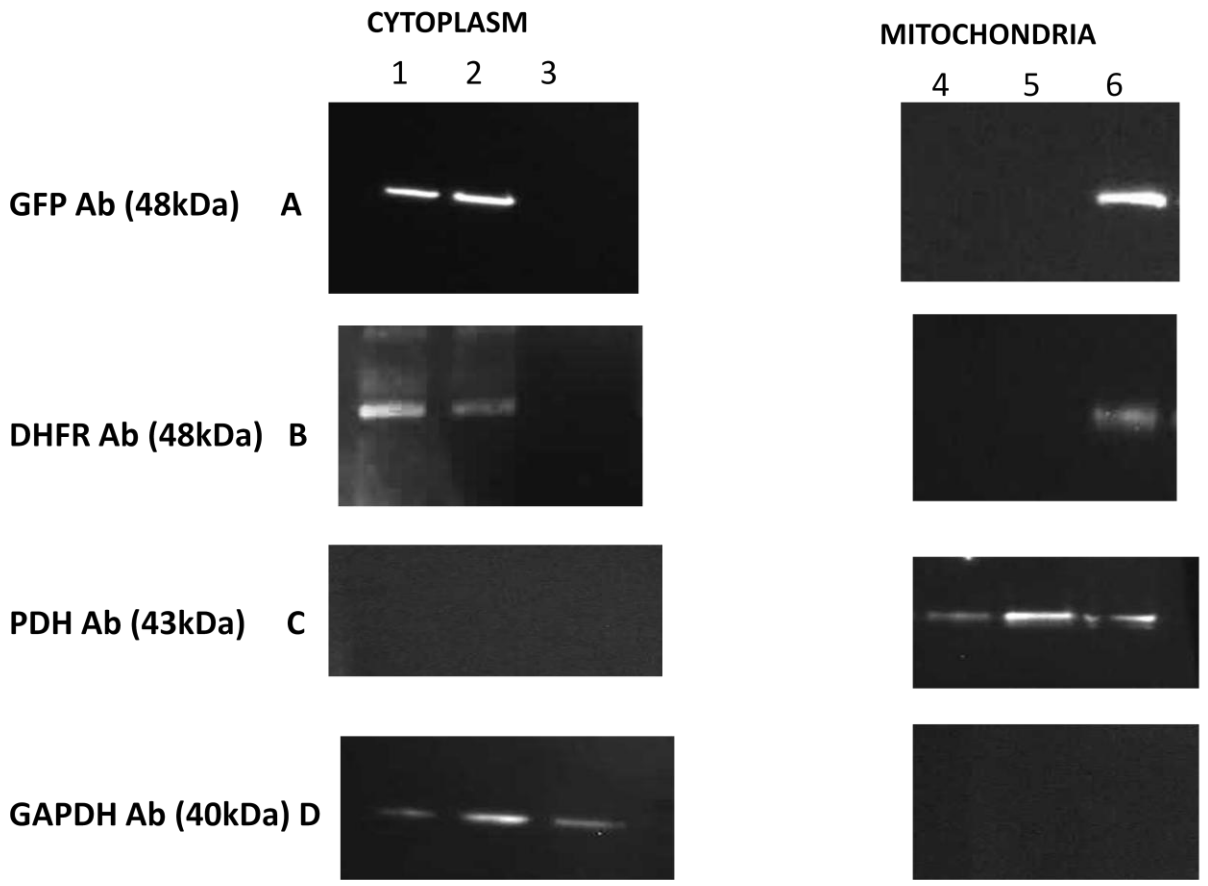


Figure 5.5 Confirmation of sub-cellular localization by western blot. The experiment was repeated and the mitochondria and cytoplasm fractions were isolated and analyzed by western blot. Lane 1 DHFR cytoplasm, Lane 2 mutant DHFRL1 cytoplasm, Lane 3 DHFRL1 cytoplasm, Lane 4 DHFR mitochondria, Lane 5 mutant DHFRL1 mitochondria and Lane 6 DHFRL1 mitochondria. Blots were probed with GFP antibody (A) and DHFR antibody (B). A clear band is present for both DHFR and mutant DHFRL1 in the cytoplasm and for DHFRL1 in the mitochondria. Blots were probed with PDH (C) as endogenous control for the mitochondria. Bands present in all three samples in mitochondria but not in the cytoplasm indicating pure mitochondria was isolated. Blots were probed with GAPDH (D) as endogenous control for the cytoplasm. Bands were present in all three samples in cytoplasm fractions but not mitochondria fractions indicating pure cytoplasm was isolated.

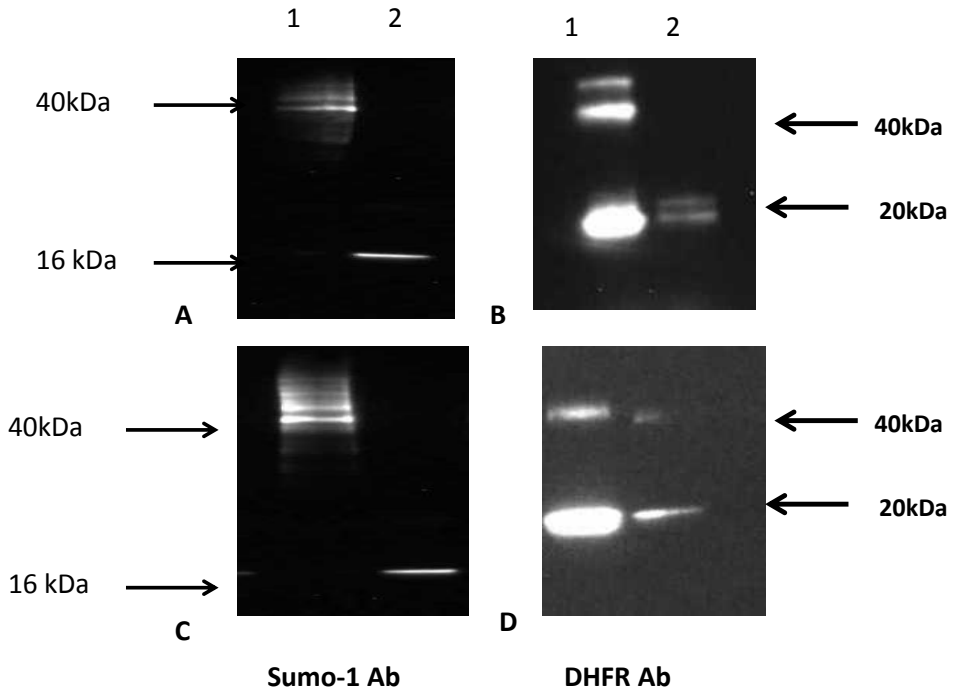


Figure 5.6 Sumoylation of DHFRL1 by *in vitro* SUMO-1 assay. In order to determine if DHFRL1 had an active sumo motif an *in vitro* SUMO-1 assay was carried out and the results analyzed by western blot. **(A)** DHFRL1 assay probed with SUMO-1 antibody. Lane 1 DHFRL1 + SUMO-1 and Lane 2 DHFRL1 + mutant SUMO-1 (negative control). **(B)** DHFRL1 assay probed with DHFR antibody. Lane 1 DHFRL1 + SUMO-1 and Lane 2 DHFRL1 + mutant SUMO-1 (negative control). The band present in both these blots at 40kDa represents sumoylated DHFRL1. The band at 16kDa in blot A is the mutant SUMO-1 protein and the band at 20kDA in blot B is free DHFRL1 protein. As a positive control the assay was repeated using DHFR protein. **(C)** DHFR assay probed with SUMO-1 antibody. Lane 1 DHFR + SUMO-1 and Lane 2 DHFR + mutant SUMO-1 (negative control). **(D)** DHFR assay probed with DHFR antibody. Lane 1 DHFR + SUMO-1 and Lane 2 DHFR + mutant SUMO-1 (negative control). Again as with the DHFRL1 assay the band at 16kDa in blot C is the mutant SUMO-1 protein and the band at 20kDA in blot D is free DHFR protein.

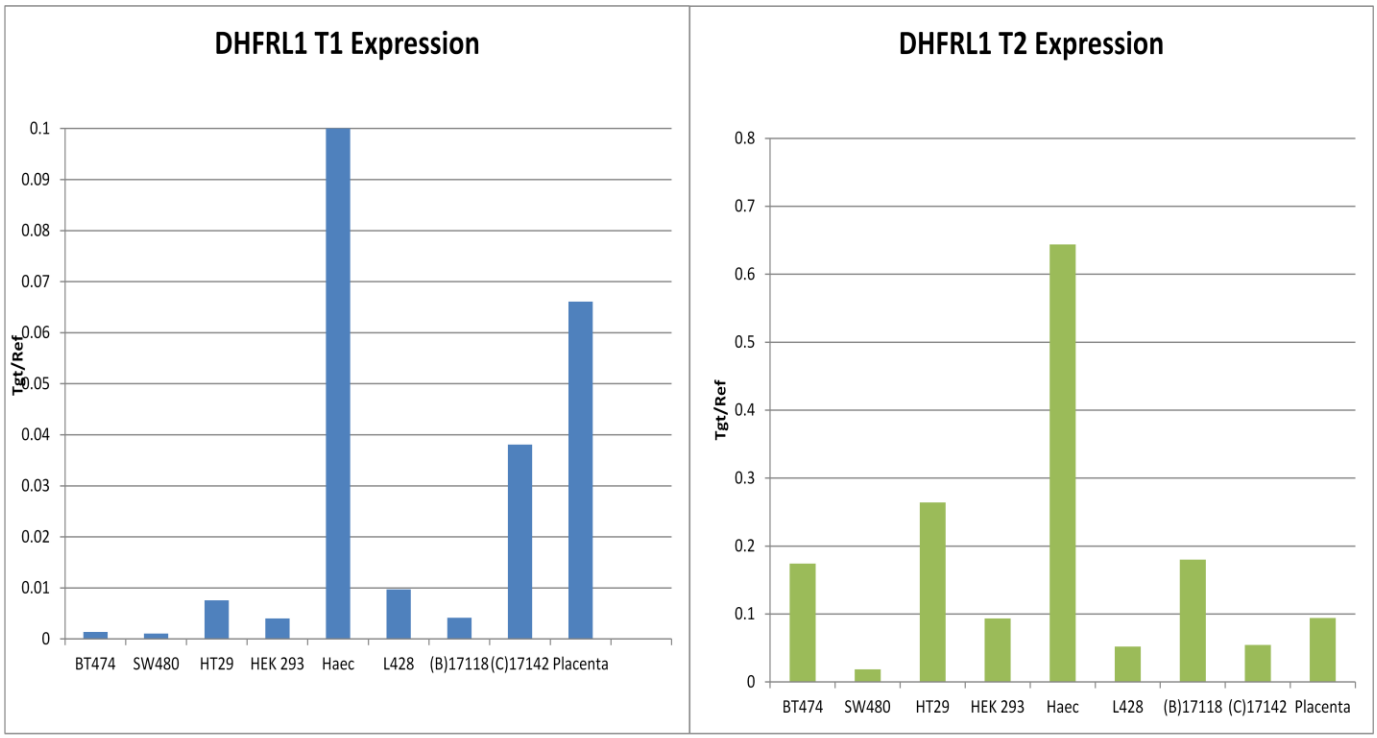


Figure 5.7 Confirmation of the expression of *DHFRL1* T1 transcript.

RT-qPCR was carried on cDNA made from RNA isolated from a number of different cell lines, using primers for the T1 transcript that were designed to distingush it from the T2 transcript. Low levels of expression were found in most cell lines with the highest level expressed in Haec cells (human aortic endothelial cells). A higher level of expression was also observed in placental RNA. All data was normalised to the endogenous control GUS. Comparing RT-qPCR data of the expression of both transcripts DHFRL1 T2 is clearly the more highly expressed transcript. In placental RNA however expression of both transcripts appears to be similar suggesting that both transcripts may have a role to play in development. **(A)** Expression of *DHFRL1* T1 transcript. **(B)** Expression of *DHFRL1* T2 transcript.

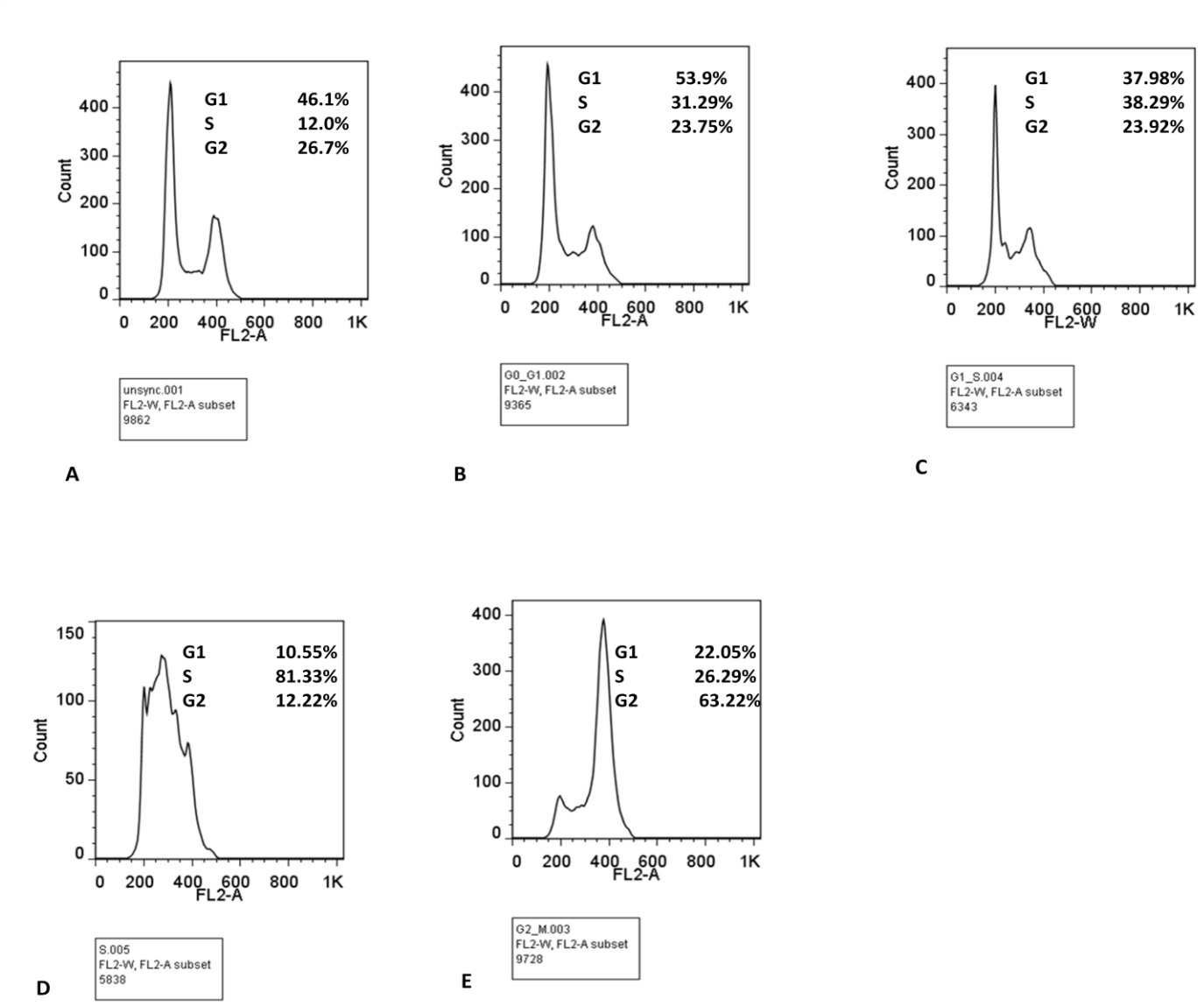


Figure 5.8 FACS analysis of cell cycle arrest. HEK 293 cells were arrested at different stages of the cell cycle. Cell cycle arrest was confirmed by flow cytometry. **A** is unsynchronized cells B is cells serum starved to arrest the cell cycle at G_0/G_1 phase. **C** is cells treated with L-mimosine to arrest cell cycle at G_1/S phase. **D** is cells treated with a double thymidine block to arrest cell cycle at S phase. **E** is the cells treated with nocadazole to arrest the cell cycle in G_2 phase.

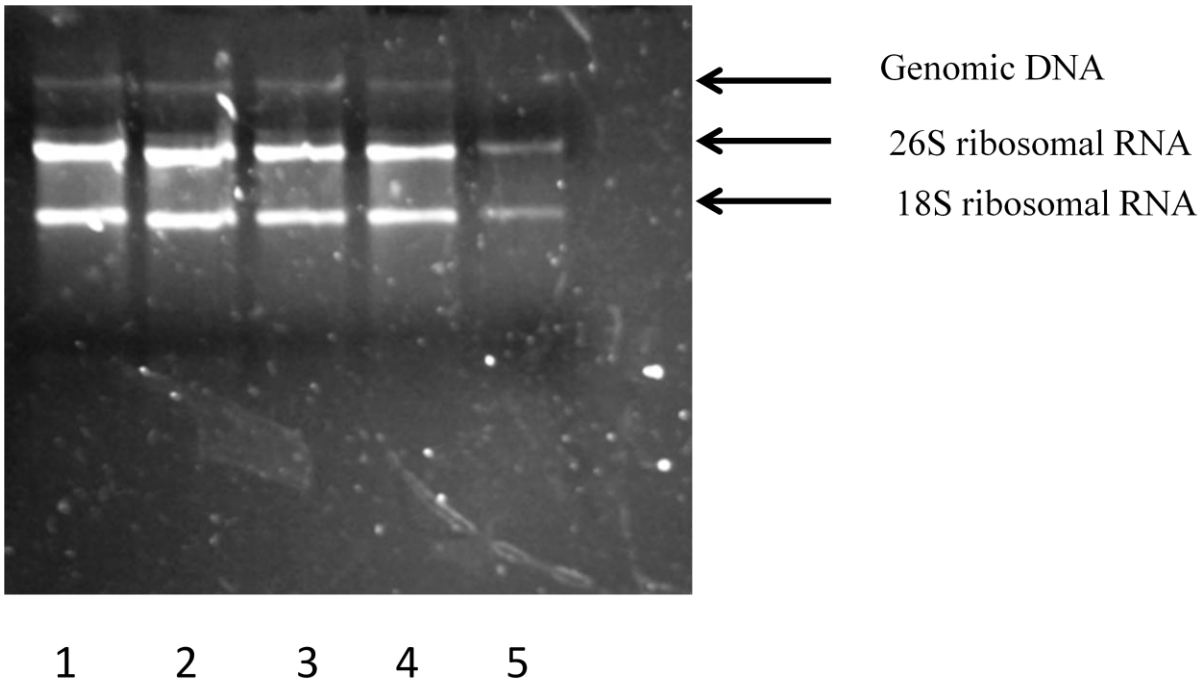


Figure 5.9 RNA isolated from cell cycle regulated HEK 293 cells. RNA was isolated from cell cycle synchronized HEK 293 cells using Bioline RNA isolation Kit. The RNA was then analyzed on a 1% agarose gel. Lane 1 unsynchronized RNA, lane 2 G_0/G_1 phase RNA, lane 3 G_1/S phase RNA, lane 4 S phase RNA and lane 5 G_2/M phase RNA. RNA is intact and not degraded and was then used to make cDNA for gene expression analysis.

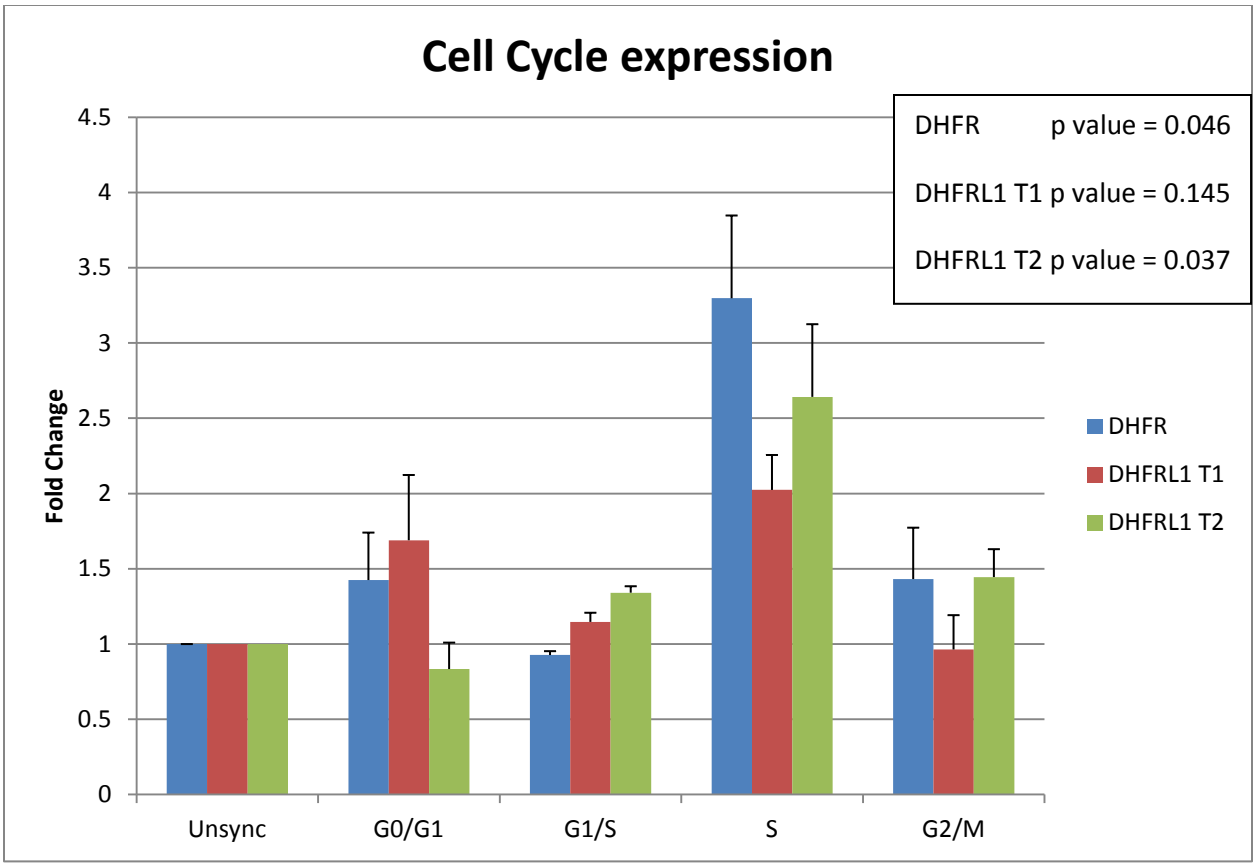


Figure 5.10 Expression levels of DHFR, DHFRL1 T1 and DHFRL1 T2 at different stages of the cell cycle. Expression levels of DHFR, DHFRL1 T1 and DHFRL1 T2 were measured by RT-qPCR and compared to the endogenous control β-actin. Levels of DHFR increased significantly during S phase of the cell cycle as did the level of DHFRL1 T2 transcript. DHFRL1 T1 transcript showed a slight increase at the S phase however expression levels of this transcript remained similar throughout the cell cycle.

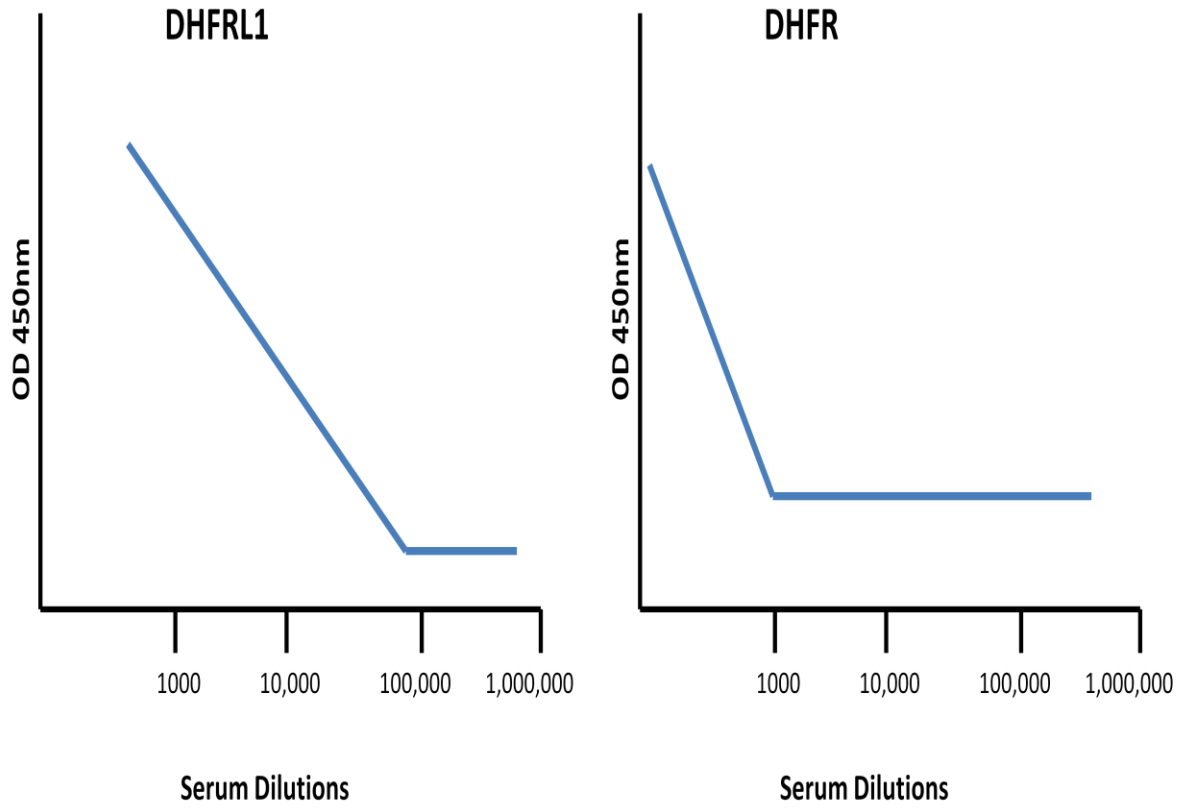


Figure 5.11 Expected results from serum titre ELISAs. Ideally the titre ELISA's should show a clear difference between wells coated with DHFRL1 and those coated with DHFR. If the serum contained a high titre of antibodies specific to DHFRL1 then the DHFRL1 graph should not level out until the high serum dilutions i.e. over 1:100,000 and the DHFR graph should level out at a much lower dilutions.

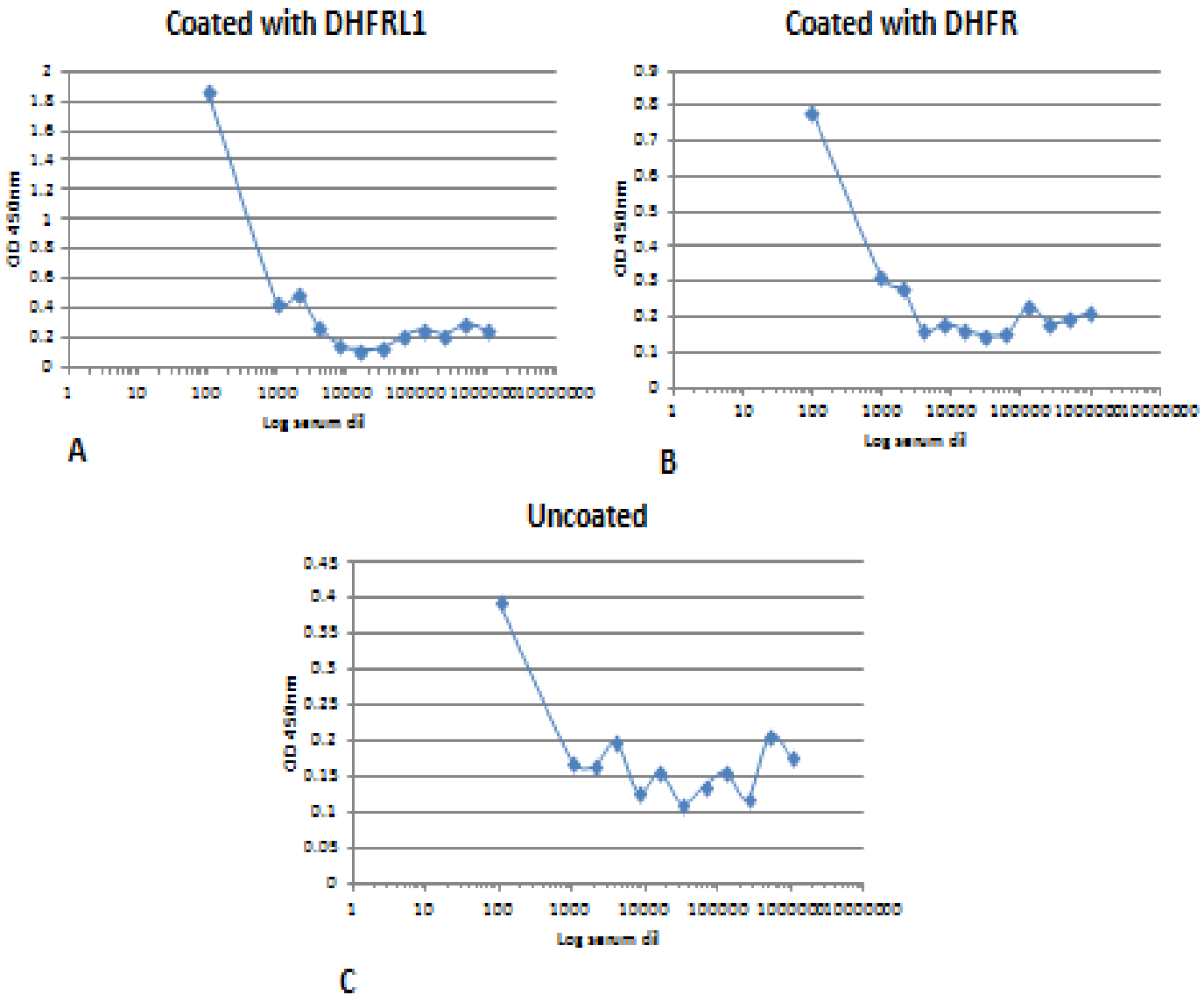


Figure 5.12 Serum titre ELISAs for mice immunized with DHFRL1 peptide. The ELISAs for both DHFR and DHFRL1 show little difference with both levelling out at around the 1:10,000 dilutions. This indicates that there is a very low number of antibodies present in the serum which can bind to either protein and also that it is unlikely that the serum contains a DHFRL1 specific antibody or of it is present it is at a very low concentration and would be difficult to separate out from the rest.

Chapter 6

Methotrexate and DHFRL1

6.1 Introduction

The ability of DHFR to reduce dihydrofolate into tetrahydrofolate, essential for DNA synthesis and methylation, has made it a clinically useful target for a number of anti-folate drug therapies. Anti-folates targeting bacterial or protozoal DHFR, mainly trimethoprim and pyrimethamine, are used to treat common bacterial or fungal diseases including malaria.[133] These drugs are generally less effective against human DHFR. A different class of drug was designed to target human DHFR with the introduction of aminopterin into the clinic in 1948,[134] followed by its more successful analogue methotrexate (MTX) in 1956.[135] Today MTX is used in the treatment of a wide range of cancers including acute lymphocytic leukaemia (ALL), [136] as well as the auto immune disease rheumatoid arthritis.[137] Figure 6.1 shows the structure of MTX.

Extensive research has been carried out to fully understand its mechanism of action and to further improve its clinical effectiveness. After administration MTX is taken up into the cells and converted into polyglutamates. It is this MTX polyglutamate that competitively binds to DHFR enzyme blocking access of the enzymes' active sites to the substrate dihydrofolate and thus depleting the tetrahydrofolate pool which in turn decreases DNA synthesis and eventually leads to cell death. [138]

Although MTX is a potent inhibitor of DHFR, drug resistance has increasingly become an issue. Resistance mechanisms so far identified include; 1) decreased accumulation due to impaired transport, 2) decreased retention as a consequence of lack of polyglutamate formation, 3) increased levels of the enzyme that hydrolyses MTX polyglutamates, 4) an increase in the levels of DHFR and 5) mutagenesis of DHFR leading to a lower binding of MTX.[139]

A number of amino acid residues of DHFR have been shown to be important for binding of MTX and mutagenesis at these sites can confer MTX resistance. One such site is Phe31. In a study carried out by Chandara et al a substitution of Phe with Gly at position 31, (F31G), showed a 100 fold decrease in affinity for MTX due to slower binding.[140] Mutagenesis of the amino acid at position 22, Leu 22, has also been proven to dramatically decrease the binding ability of MTX. [141] [142]

It was first reported in 1967 that exposure of mammalian cells to MTX led to an initial increase in DHFR activity.[143] It has since been discovered that this can be the result of a number of

different actions including genomic amplification of the DHFR gene.[144] In some cell lines the genomic amplification is stable whereas in others it is lost after exposure to MTX is removed.[145] A more recent study carried out by Morales et al showed that cells which reverted back to a low copy number of the DHFR gene were likely to respond to a second dose of MTX and had a decreases ability to generate resistance.[146] There is also evidence to suggest that increased levels of DHFR after exposure to MTX can be the result of increased stimulation of the promoter which in turn increases transcription of the DHFR gene[147]

The increase in DHFR levels in response to exposure to MTX as a result of gene amplification is usually permanent however thee is also a transient mechanism to increase expression. DHFR can auto-regulate its own translation by binding to its own mRNA to prevent that mRNA from becoming translated.[148] The initial response to MTX is to disrupt this mRNA: protein complex releasing the protein leading to an increase in DHFR activity.[149]

As MTX is a potent inhibitor of DHFR it is essential that we understand what effect it may have on DHFRL1. As MTX is a folate analogue that binds to the enzyme in place of the substrate it is possible that as DHFRL1 has a lower affinity for dihydrofolate it will also have a lower affinity for MTX. However this needs to be addressed experimentally. MTX is not known to enter the mitochondria where DHFRL1 has a strong presence (Section 4.2), so while it is possible that MTX may be a good inhibitor of DHFRL1 it may simply never come into contact with the enzyme. The biological significance of the response to MTX by DHFRL1 may be more relevant to the translation regulation mechanism. DHFRL1 has also been shown to be capable of binding its own mRNA and that of DHFR to regulate translation.[150] The impact, if any, MTX has on this mRNA: protein complex may significantly alter both DHFR and DHFRL1 protein levels within the cell.

The aim of this chapter is to assess the response of DHFRL1 to MTX. This will include:

- Calculating the Ki, binding affinity, of MTX for purified recombinant protein.
- Measuring enzyme activity levels of DHFRL1 in response to MTX in a cell culture model
- Investigating the impact of MTX on the binding of DHFRL1 protein to both its own mRNA and that of DHFR.

RESULTS

6.2 Determining the disassociation constant, Ki, of MTX for DHFRL1

There are three distinct categories of enzyme inhibition by a target molecule. They are:

1. **Competitive Inhibition –** this is where the inhibitor molecule is highly similar to the substrate molecule and competes with the substrate to bind to the enzymes active sites. Once the inhibitor is bound the enzyme is unavailable to the substrate and all catalytic activity is stopped.
2. **Non-competitive Inhibition -** this is where the inhibitor molecule binds to a site other than the enzymes active site. While the enzyme can still bind substrate this inhibitor molecule modifies the enzyme so the catalytic process is not as efficient.
3. **Un-competitive Inhibition –** this is where the inhibitor molecule binds to the complex formed by the enzyme and substrate. This molecule will only target enzymes that have substrate already bound, which prevents the enzyme from converting substrate into product.

MTX acts as a competitive inhibitor of DHFR. An essential first step was to determine if MTX is also a competitive inhibitor of DHFRL1. A number of enzyme assays were carried out varying the concentration of substrate dihydrofolic acid while keeping the inhibitor MTX constant. This was repeated for a number of different concentrations of MTX. Competitive inhibition works when the inhibitor molecule (MTX) binds to an enzymes active sites making the enzyme unavailable for catalysis, in other words if the enzyme is bound by inhibitor it has a lower affinity for its substrate and as a result has a higher Km for the substrate. This is graphically depicted using Line-Weaver Burke plots which remain linear but as the concentration of MTX increases so too does the Km. The data was plotted on Line-Weaver Burke graphs. The resulting plots of each concentrations of MTX tell us if the drug acts as a competitive or non-competitive inhibitor. Figure 6.2 shows the Line-Weaver Burke plots for both DHFR and DHFRL1. Both enzymes display competitive inhibition of MTX although DHFRL1 did require higher concentrations of the drug to achieve inhibition.

Once competitive inhibition was established a Ki of MTX for DHFRL1 could be determined. The Ki is the disassociation constant and is similar to the Km value in that it is a measurement of the affinity of the enzyme for the drug. Once again the higher the Ki value the lower the affinity.

A graphic depiction of the calculation of Ki is shown in Figure 6.3. Briefly the Km for dihydrofolic acid over a range of MTX is determined as outlined in Section 3.5. These Km values are then plotted against the inhibitor concentration to give a linear plot. The slope of this line is equal to the Km at 0pM MTX divided by the Ki (slope = Km/Ki). Ki values for both DHFR and DHFRL1 were determined using this method. Results are shown in Table 6.1. DHFR had a Ki value of 3.15pM which corresponds to the values in the literature. [151] DHFRL1 had a significantly higher value of 60.14 pM indicating a much lower affinity for methotrexate.

6.3 Disruption of auto-regulation

The mechanism of auto-regulation of both DHFR and DHFRL1, discussed in more detail in Chapter 4, involves the binding of the protein to the mRNA preventing that mRNA from being translated. An initial response of treatment with MTX is an increase in the levels of dihydrofolate reductase activity. This observation was first made in mammalian cells in 1967 [140] and further investigation led to the discovery that one of the reasons for the increase in DHFR activity is due to the fact that MTX disrupts the DHFR: mRNA binding complex releasing the protein and allowing the mRNA to be translated into protein.[146] The effect of MTX on DHFRL1: RNA binding was examined using a mammalian cell culture model and also by conducting REMSA binding experiments.

6.3a Response of DHFRL1 to MTX in mammalian cells

For these experiments the Chinese hamster ovary cells stably transfected with either DHFR or DHFRL1, created for the complementation study outlined in Section 3.4, were used. These cells were chosen as the parent cell line CHO DG44 does not have any dihydrofolate reductase activity therefore any response observed will be as a result if the human DHFR or DHFRL1 inserted into the cells.

The concentration of MTX used was determined by treating cells with a range of MTX from 0-10μM. An MTT assay carried out as outlined in Section 2.2.2b was used to determine what concentration of MTX would not be toxic to the cells, (Figure 6.4). A concentration of either 2μM or 5μM MTX was used to treat cells. After treatment with MTX protein and RNA was isolated as outlined in Section 2.4.1a and Section 2.3.3 respectively, at 0hr, 2hr, 4hr, 6hr and 24hr to see the initial response of DHFRL1 to MTX. The CHO DHFR cells treated with MTX acted as a positive control for the experiment.

The isolated protein was analyzed both by enzyme assay as described in Section 2.4.5 and Western blot as described in Section 2.4.4. As expected the enzyme assays for DHFR for both 2μM and 5μM MTX treatment showed an increase in enzyme activity 4 hours after exposure to MTX. This result was confirmed by western blot which showed an increase in DHFR protein after 4 hours exposure to MTX. See Figures 6.5 and 6.6 for enzyme assay and western blot results respectively.

The enzyme assays for DHFRL1 protein show that there was no increase in dihydrofolate reductase activity after treatment with 2μM MTX however an increase in enzyme activity was observed at 2 hrs exposure to 5μM MTX. Again these enzyme results were confirmed by Western blot which showed an increase in protein for 5μM MTX but not for 2μM MTX treatment. See Figures 6.5 and 6.6 for enzyme assay and western blot results respectively.

The isolated RNA was analyzed by RT-qPCR as outlined in Section 2.3.6. The RNA levels for both DHFR and DHFRL1 remain relatively unchanged after exposure to MTX compared to the increase in enzyme activity. DHFR had a 1 fold increase in RNA expression at 2 hrs after exposure to 2μM MTX however RNA levels returned to normal by 4 hrs. DHFRL1 mRNA levels after exposure to 2μM MTX showed a slight decrease of approx 0.5 fold. For the 5μM MTX treatment DHFRL1 mRNA levels increased by 0.5-1 fold for the 2 hr and 4 hr time points. See Figure 6.7 for RT-qPCR data.

A comparison of RNA expression and enzyme activity for both DHFR and DHFRL1 at both concentrations of MTX is shown in Figure 6.8. The results indicate that while the increase in expression levels may contribute a small proportion of the increased enzyme activity it is most

probable that the majority of increased enzyme activity is as a result of the disruption of the protein: RNA binding complex.

6.3b The effect of MTX on DHFRL1 RNA binding

The mammalian cell culture experiment showed that similar to DHFR, the initial response of DHFRL1 to MTX is an increase in dihydrofolate reductase activity however a higher concentration of MTX is required before this response is observed. In order to determine if this increase in enzyme activity is due to disruption of DHFRL1: mRNA binding complex REMSA experiments outlined in Section 2.5 and section 4.2 were repeated with either 2µM or 5µM MTX added to the reactions. The following reactions were set up:

1. Labelled RNA only
2. Labelled RNA + protein
3. Labelled RNA + protein + unlabelled RNA
4. Labelled RNA + protein + MTX

The results for DHFRL1 protein bound to both DHFR and DHFRL1 RNA incubated with 2µM MTX are shown in Figure 6.9a. The results clearly show that 2µM MTX does not disrupt the binding to either DHFR or DHFRL1 RNA. When DHFR protein bound to both DHFR and DHFRL1 RNA was incubated with 2µM MTX disruption of binding is observed as shown in Figure 6.9b. When the concentration of MTX was increased to 5µM a clear disruption of binding was observed for DHFRL1 protein bound to both DHFR and DHFRL1 RNA, results shown in Figure 6.10a. As expected 5µM MTX also disrupted binding of DHFR protein bound to both DHFR and DHFRL1 RNA, results shown in Figure 6.10b.

From these experiments it can be concluded that a higher concentration of MTX is required in order to disrupt DHFRL1 binding to either its own mRNA or that of DHFR.

6.4 Discussion

Methotrexate is a potent inhibitor of DHFR which is used in the treatment of a number of diseases such as leukaemia and rheumatoid arthritis. MTX is a folate analogue which competes with the natural substrate of DHFR, dihydrofolate, to bind to the enzymes' active sites preventing the substrate from accessing those sites leading to the downstream effect of preventing DNA synthesis. As MTX is a commonly used inhibitor of DHFR it is essential that the response of DHFRL1 to MTX is also characterized.

As previously stated MTX is a folate analogue and a number of the amino acids required to bind dihydrofolate are also required to bind MTX. As discussed in detail in Chapter 3 DHFRL1 does have enzyme activity however the Km for the substrate is much higher when compared to DHFR indicating a much lower binding affinity for the substrate which is most likely due to the W24R substitution. This lower binding affinity is also exhibited by DHFRL1 for MTX which has a Ki value that is almost 20 fold higher than for DHFR. This lower binding affinity potentially makes MTX a much less potent inhibitor of DHFRL1.

As discussed in detail in Chapter 4 DHFR has the ability to auto-regulate its own translation by the binding of the protein to its own mRNA molecules. Also detailed in Chapter 4 is the ability of DHFRL1 protein to bind to both DHFR and DHFRL1 mRNA molecules. In mammalian cell culture one of the initial responses to MTX treatment is an increase in DHFR enzyme activity as MTX disrupts the RNA: protein binding complex realising the protein and allowing the mRNA to be translated. [152] As DHFRL1 displays RNA binding capabilities, but also shows a lower affinity for MTX, it is important to establish if MTX can disrupt DHFRL1 protein binding to both its own mRNA and that of DHFR.

Using a mammalian cell culture model with cells stably transfected with DHFRL1, parent cells do not express endogenous DHFR, the initial response of DHFRL1 at both RNA and protein level were observed. When cells were treated with 2μM of MTX levels of DHFRL1 enzyme activity only increased by 0.2 fold after 2 hours and returned to baseline by 4 hours and even displayed a slight decrease at 6 hours and 24 hours. RNA levels showed no increase after 2μM treatment, in fact a slight decrease in RNA levels was observed. Comparing these results with those obtained from stably transfected DHFR cells, which displayed a 5 fold increase in enzyme

activity after 4 hours with levels remaining above baseline at 6 and 24 hours, while RNA levels showed a slight increase at 2 hours but returned to baseline by 4 hours. These results indicate that 2μM MTX can disrupt the DHFR protein: RNA binding complex however it is not able to disrupt the DHFRL1 protein: RNA binding complex.

Taking into consideration DHFRL1's lower binding affinity for MTX the experiments were repeated using the increased concentration of 5μM MTX. Again DHFR displays an increase in enzyme activity after 4 hours however the increase is not as high compared to 2μM MTX. It is possible that for these cells 5μM MTX is becoming cytotoxic and the cells are starting to die off. At 5μM MTX DHFRL1 cells show a 2.5 fold increase in enzyme activity with enzyme activity remaining above baseline until 24 hour time point. The RNA levels show a slight increase at the 2 and 4 hours however the increase is not as high as the enzyme activity. This would indicate that while it may be possible that some of the increased enzyme activity is due to increased expression it is unlikely to have contributed to all of the increased enzyme activity.

The cell culture experiments correlate with the Ki data indicating that a higher concentration of MTX is required to see a response for DHFRL1 compared to DHFR. These experiments also appear to show that a higher concentration of MTX can also disrupt the DHFRL1 protein: RNA biding complex. To confirm that MTX can disrupt this binding complex the REMSA experiments outlines in Section 4.2 were repeated to include either 2μM or 5μM MTX. As in the cell culture experiment DHFR showed a response to both 2μM and 5μM MTX, with disruption to the binding of both DHFR and DHFRL1 mRNA. DHFRL1 did not show a response to 2μM of MTX with binding to both DHFR and DHFRL1 mRNA undisrupted. DHFRL1 protein however did show a response when MTX concentration was increased to 5μM, binding to both DHFR and DHFRL1 mRNA was disrupted at this concentration of MTX. An overview of the molecular response of DHFR and DHFRL1 to MTX is shown in Figure 6.11 and Figure 6.2 respectively.

6.4.1 DHFRL1 as a therapeutic target

Due to the potency of MTX to DHFR it is important to also understand the response of DHFRL1 to MTX. However a significant difference between DHFR and DHFRL1 is the localisation of DHFRL1 to the mitochondria while DHFR is primarily located in the cytoplasm. This difference of location within the cell is important in relation to the response to MTX as MTX does not enter the mitochondria and therefore is unlikely to have adequate access to DHFRL1. However a recent study carried out by Pereira et al[153] may provide a solution to overcome this challenge. A mitochondrial targeting vector was created from a class of peptides known to be capable of penetrating the mitochondria. The N terminal of the targeting vector was then conjugated to the MTX molecule and the resulting localisation experiments confirmed that the MTX – peptide conjugate was present in the mitochondria.

The concept of modifying an existing anti-folate drug to target the mitochondria widens the spectrum of potential drug targets for DHFRL1. As the mitochondria are an established centre of folate metabolism a number of anti-folate drug therapies have been designed so they can target and enter the mitochondria. An example of one such drug is DATHF (5-deazaacyclotetrahydrofolate) an inhibitor of glycinamide ribonucleotide formyl transferase (GARFT) which results in the disruption of *de novo* purine biosynthesis.[154] However an obstacle in using this class of drug is that while they can enter the mitochondria they were not designed to inhibit DHFR and as a result their potency against DHFRL1 may be greatly diminished.

A number of drugs already exist which specifically target DHFR aside from MTX. These include trimethoprim used in the treatment of bacterial infections[155] it was first introduced into the clinic in 1962 as a combination therapy with sulphonamides, however from the 1970s onwards it has been used on its own.[156] Pirtrexim is a lipid soluble anti-bacterial analogue of MTX which also targets DHFR [157] and has been used in a clinical setting to treat immuno-compromised patients who develop bacterial infections. This drug differs from MTX in that is cannot be polyglutamated and crosses membranes by passive diffusion , however *in vitro* studies have shown it to be a potent as MTX in inhibiting DHFR. [158] Although originally developed as an anti-bacterial agent, piritrexim has also been used as an effective chemotherapeutic against urothelial cancer.[158] Other antifolate drugs that specifically target DHFR include trimetrexate, aminopterin and pyrimethamine. [144]

Although DHFR is one of the major targets for anti folate drug therapies some new generation drugs have been developed which target multiple enzymes in the folate pathway. One such drug is pemetrexed which targets DHFR, TS, GARFT, aminoimidazole carboxamide formyl transferase (AICARTT) and C1 tetrahydrofolate synthase. [159] It is a chemotherapeutic drug that is used in the treatment of a wide range of cancers including small cell lung cancer, correctal cancer, breast cancer and cervical cancer. As well as having a broad clinical spectrum pemetrexed has also shown a response in patients that are resistant to other antifolate therapies. [159]

The list of anti-folate therapies available in the clinic is extensive, and with the advantage of the high similarity between DHFR and DHFRL1 it is possible that one or more of these available drugs may be a potent inhibitor of DHFRL1. Although the presence of DHFRL1 in the mitochondria distinguishes it from DHFR, the concept of targeting specific drugs to the mitochondria does not limit potential drug targets to those already capable of crossing the mitochondrial membrane.

Any potential target or targets of DHFRL1 will need to be extensively investigated both *in vitro* and *in vivo* to determine potency and cytotoxicity. If a potent inhibitor of DHFRL1 can be identified and characterized it may be possible to develop a dual target strategy whereby DHFR is the target in the cytoplasm and DHFRL1 the target in the mitochondria. A multiple target strategy such as this may improve the clinical outcomes for numerous diseases whereby DHFR is already a therapeutic target.

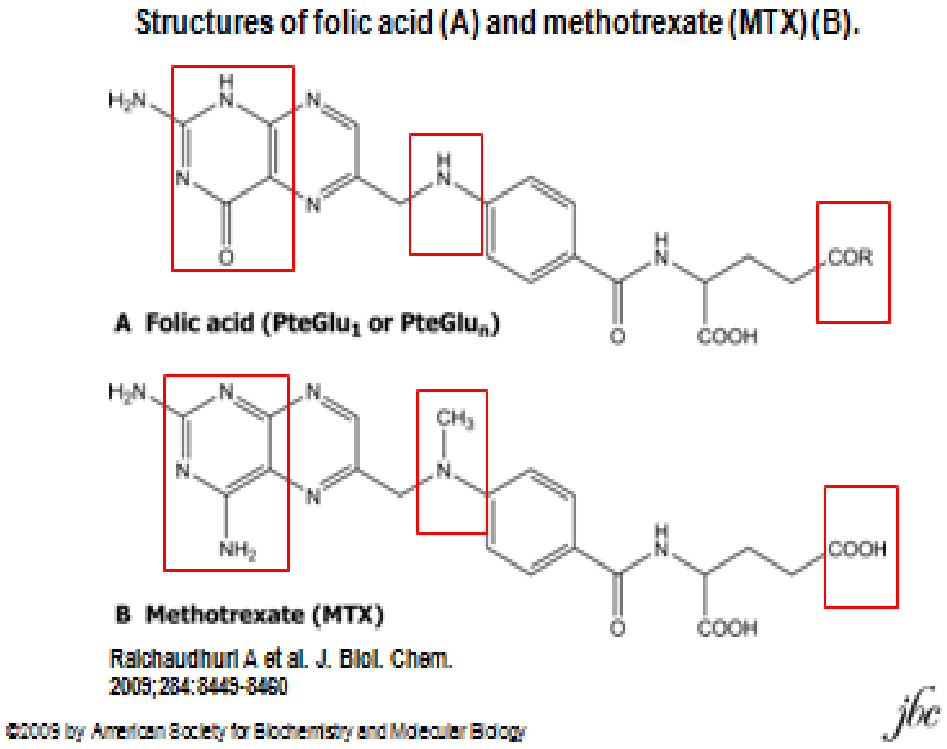


Figure 6.1 Structure of folic acid and anti-folate drug methotrexate. Image taken from Raichaudhuri A *et al. J. Biol. Chem.* (2009) **284**:8449 -8460

MTX acts as a competitive inhibitor of DHFR, meaning it competes with the substrate to bind to the enzymes active sites. Therefore MTX has a highly similar structure to the substrate with only a few variations to allow it to bind to the enzymes active sites. The differences in MTX structure are highlighted in the red boxes.

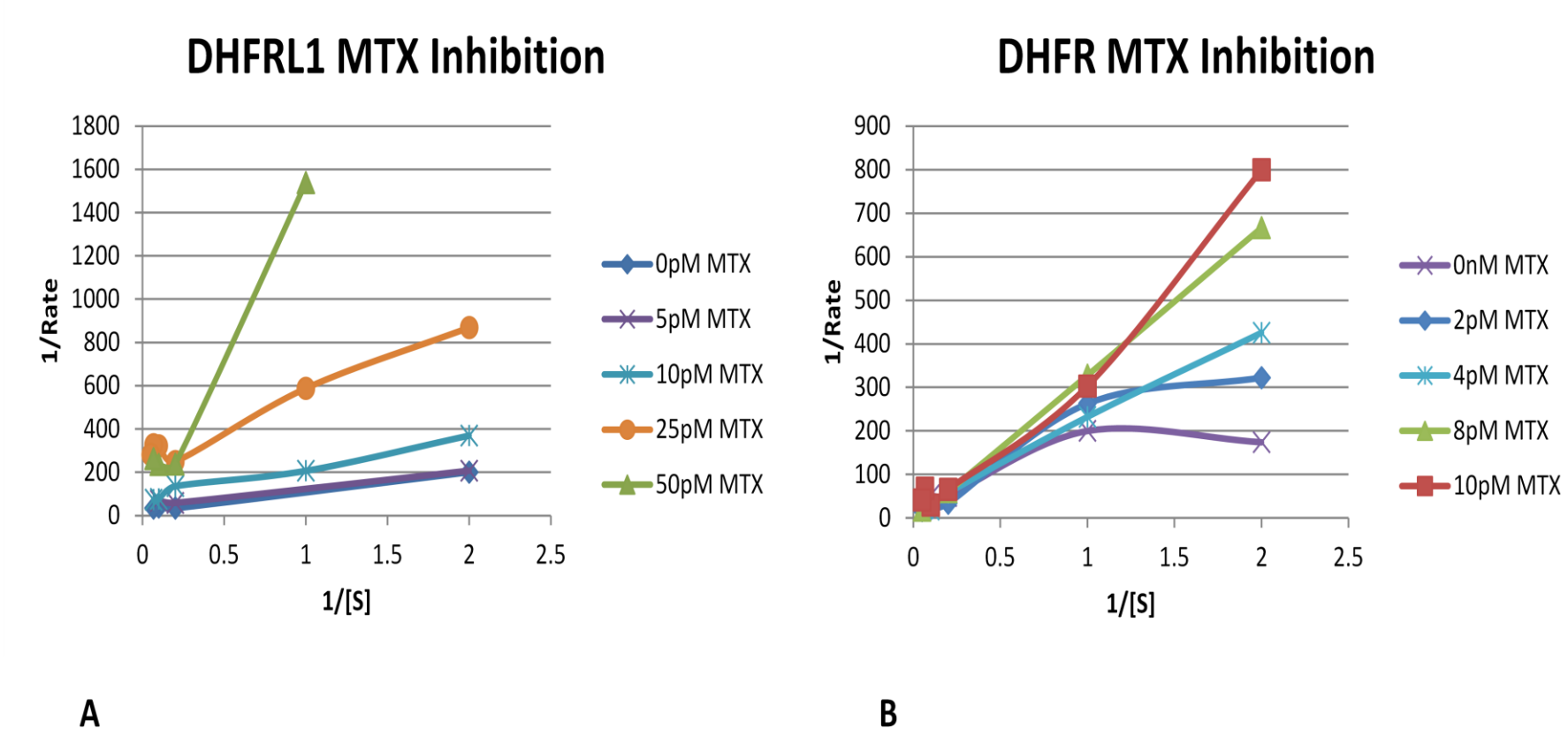


Figure 6.2 Competitive inhibitions of DHFR and DHFRL1 by MTX. Competitive inhibition works when the inhibitor molecule (MTX) binds to an enzymes active sites making the enzyme unavailable for catalysis, in other words if the enzyme is bound by inhibitor it has a lower affinity for its substrate and as a result has a higher Km for the substrate. This is graphically depicted using Line-Weaver Burke plots which remain linear but as the concentration of MTX is increased so too does the Km. Increasing concentrations of MTX shows that the drug acts as a competitive inhibitor for both DHFRL1 **(A)** and DHFR **(B)**. DHFRL1 required a higher concentration of MTX to achieve inhibition compared to DHFR.

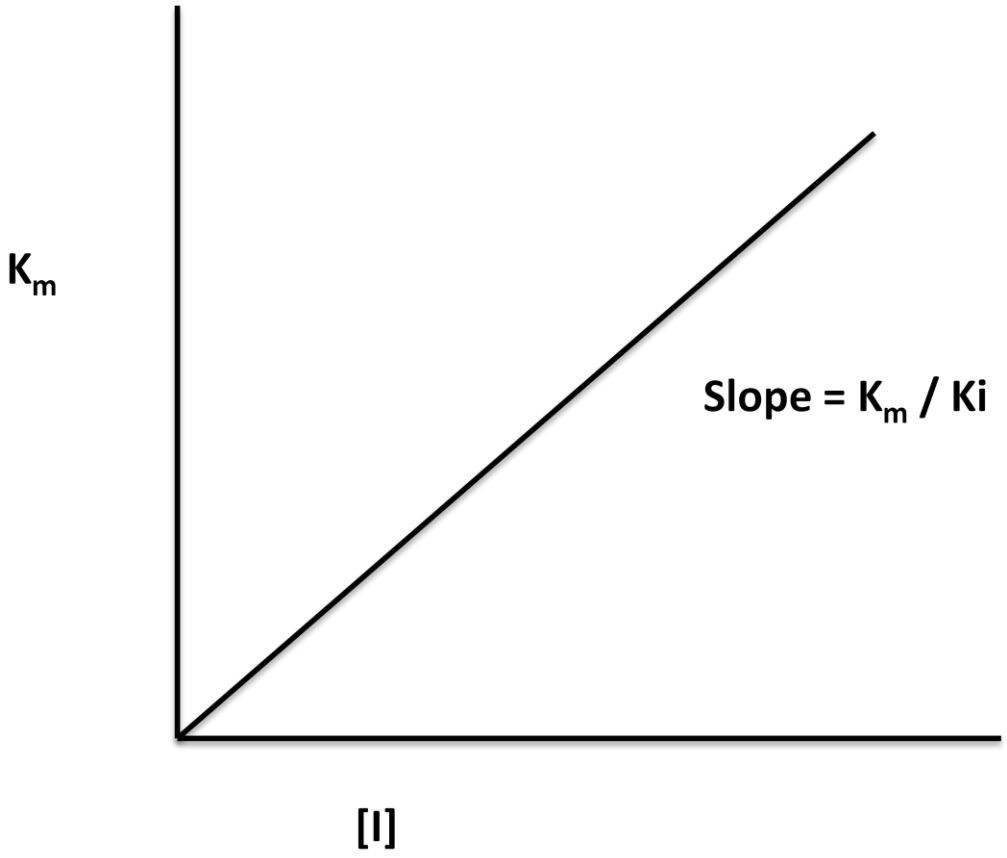


Figure 6.3 Determination of Ki. If the measurement of K_m is repeated at different concentrations of the inhibitor, the Ki can be determined from the slope of the line and the true K_m of the enzyme where the concentration of I is 0 i.e. the intercept of the line.

Table 6.1 Ki of MTX for both DHFR and DHFRL1

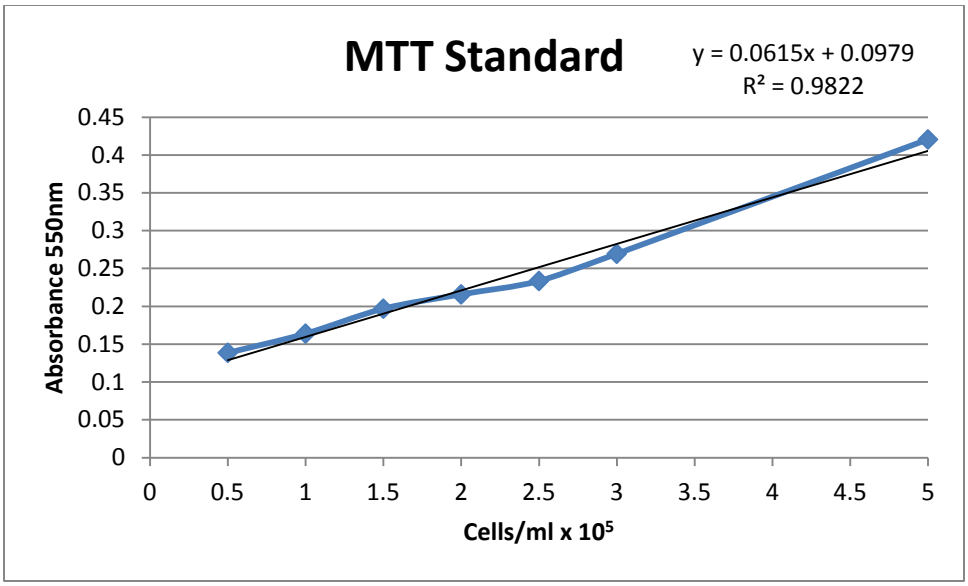
Enzyme	Ki pM*†
DHFR	3.15 ± 0.6 ¶
0-10pM §	
DHFRL1	60.14 ± 0.3 ¶
0-200pM §	

*At constant 50 μM NADPH

† 0.2-20μM Dihydrofolic Acid

§ Methotrexate concentration range

¶ Standard Error



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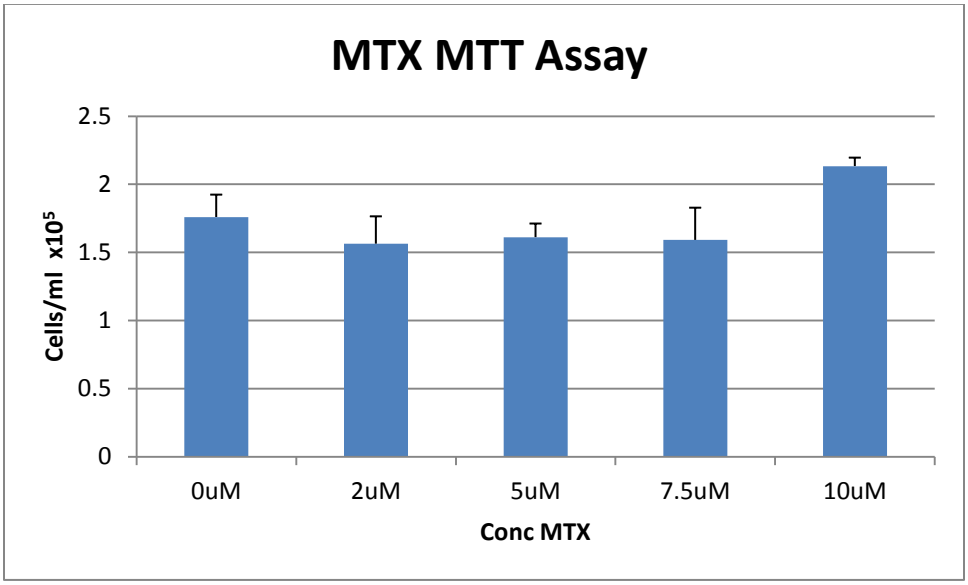
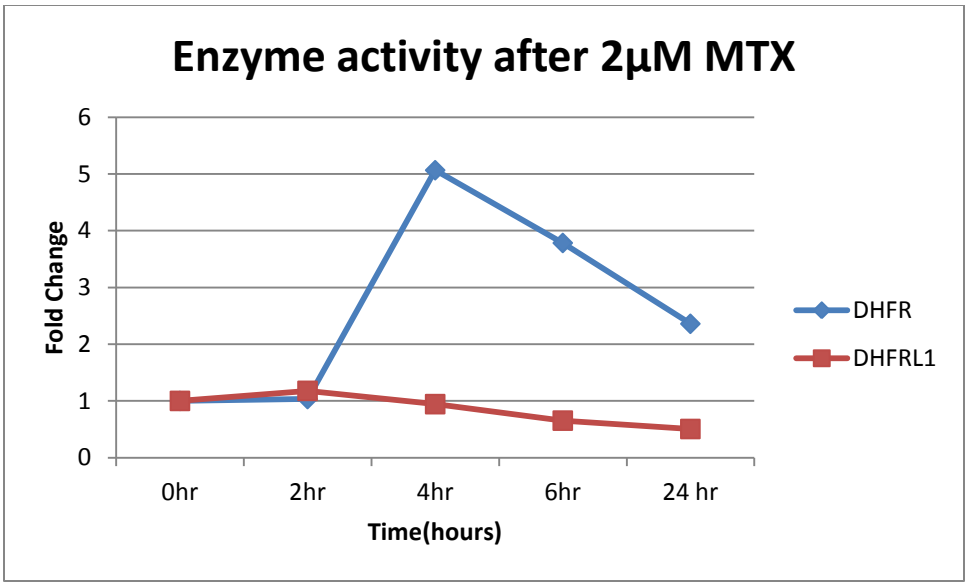


B

Figure 6.4 MTT Assay to determine toxicity of MTX. (A) A MTT assay is performed by setting up a standard curve of known concentrations of cells and reading the absorbance at 550nm after labelling reagent is added. **(B)** This standard curve was then used to determine the number of cells present after treatment with varying concentrations of MTX to determine the toxicity of the drug to the cells.



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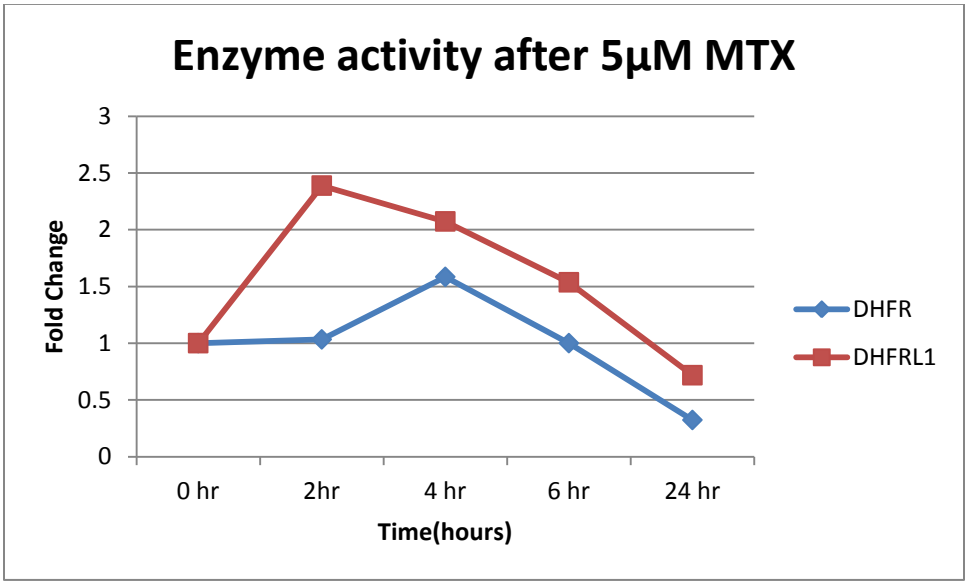

B

Figure 6.5 Measurement of enzyme activity after MTX treatment. Protein was isolated from cells at various time points after MTX treatment and enzyme activity was measured. As expected the DHFR cells showed an increase in enzyme activity after treatment with 2µM MTX. DHFRL1 cells showed no increase in enzyme activity after treatment with 2µM MTX however a 2.5 fold increase in activity was observed in DHFRL1 cells 2 hours after treatment with 5µM MTX.

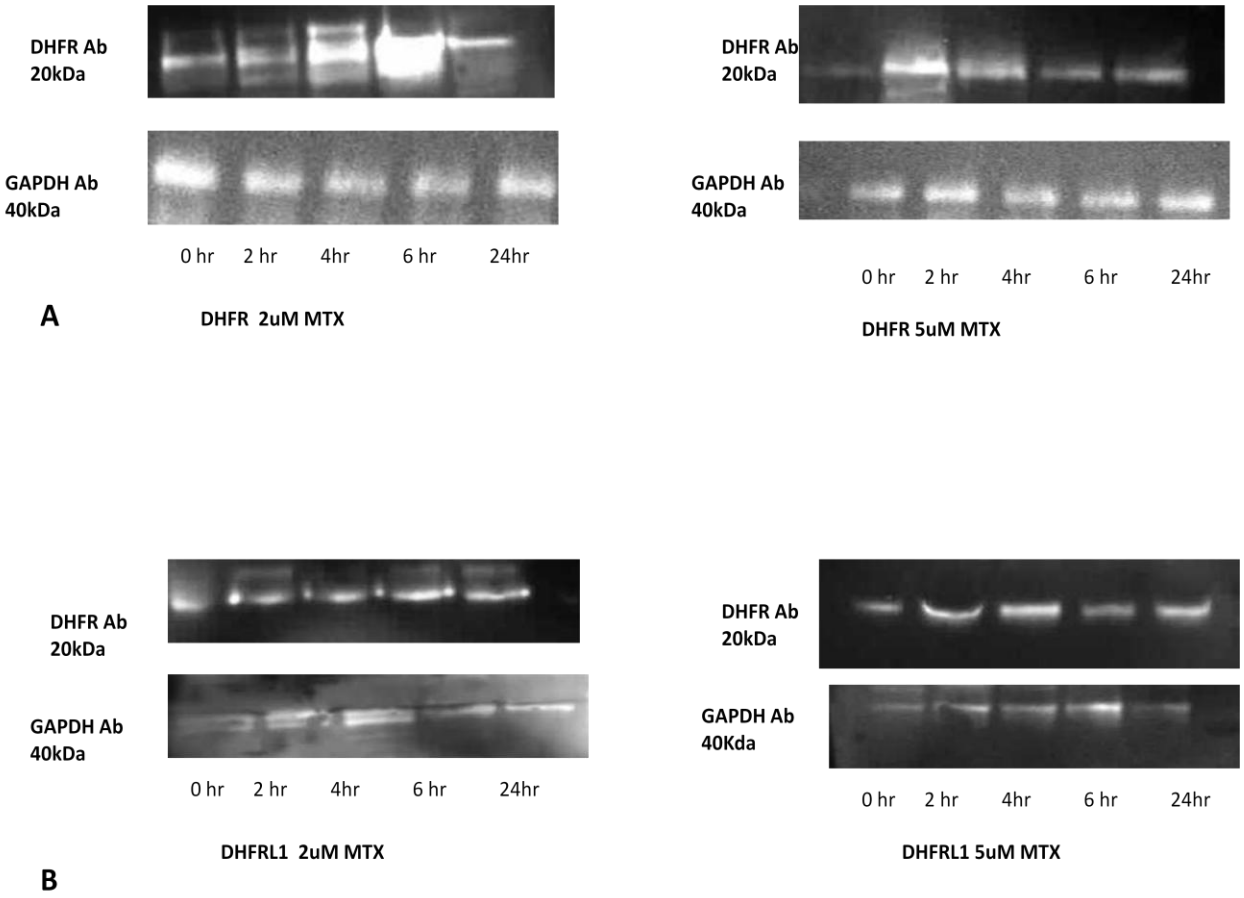

Figure 6.6 Western Blots of protein isolated after treatment with MTX. Protein was isolated from cells at various time points after MTX treatment and a western blot was carried out to confirm results from enzyme assays. **(A)** As expected the DHFR cells showed an increase in protein after treatment with both 2µM and 5µM MTX. **(B)** DHFRL1 cells showed no increase in protein after treatment with 2µM MTX however an increase in protein levels was observed in DHFRL1 cells after treatment with 5µM MTX. These results correlate with the enzyme assay data.

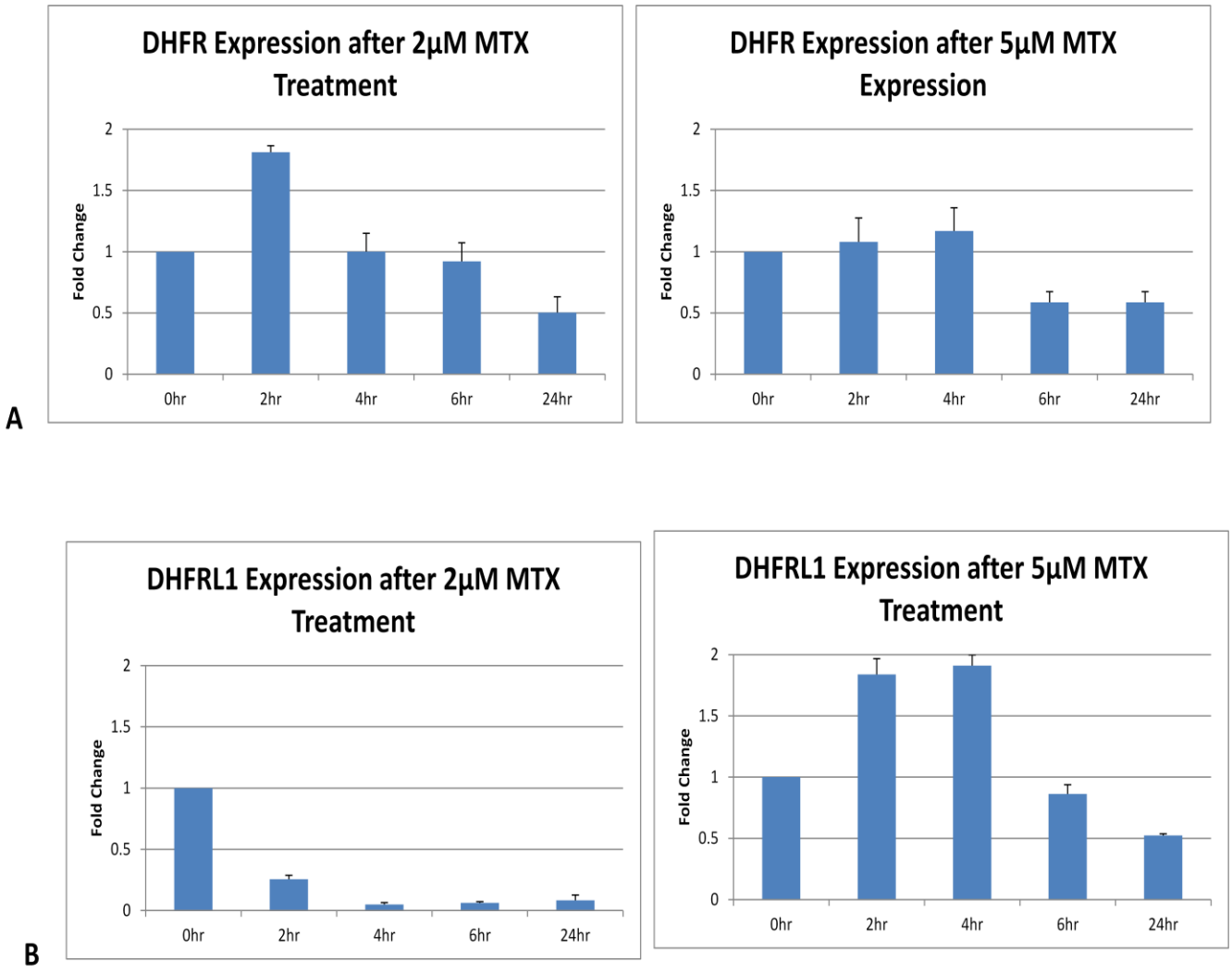

Figure 6.7 Expression of DHFR/DHFRL1 after MTX treatment. The RNA levels of DHFR/DHFRL1 at varying time points after MTX treatment was measured by RT-qPCR. **(A)** DHFR RNA levels show a slight increase at 2 hours after 2µM MTX treatment and **(B)** DHFRL1 RNA levels also show an increase after exposure to 5µM MTX. The increases in RNA levels are much lower than the increases in enzyme activity observed for the same time points.

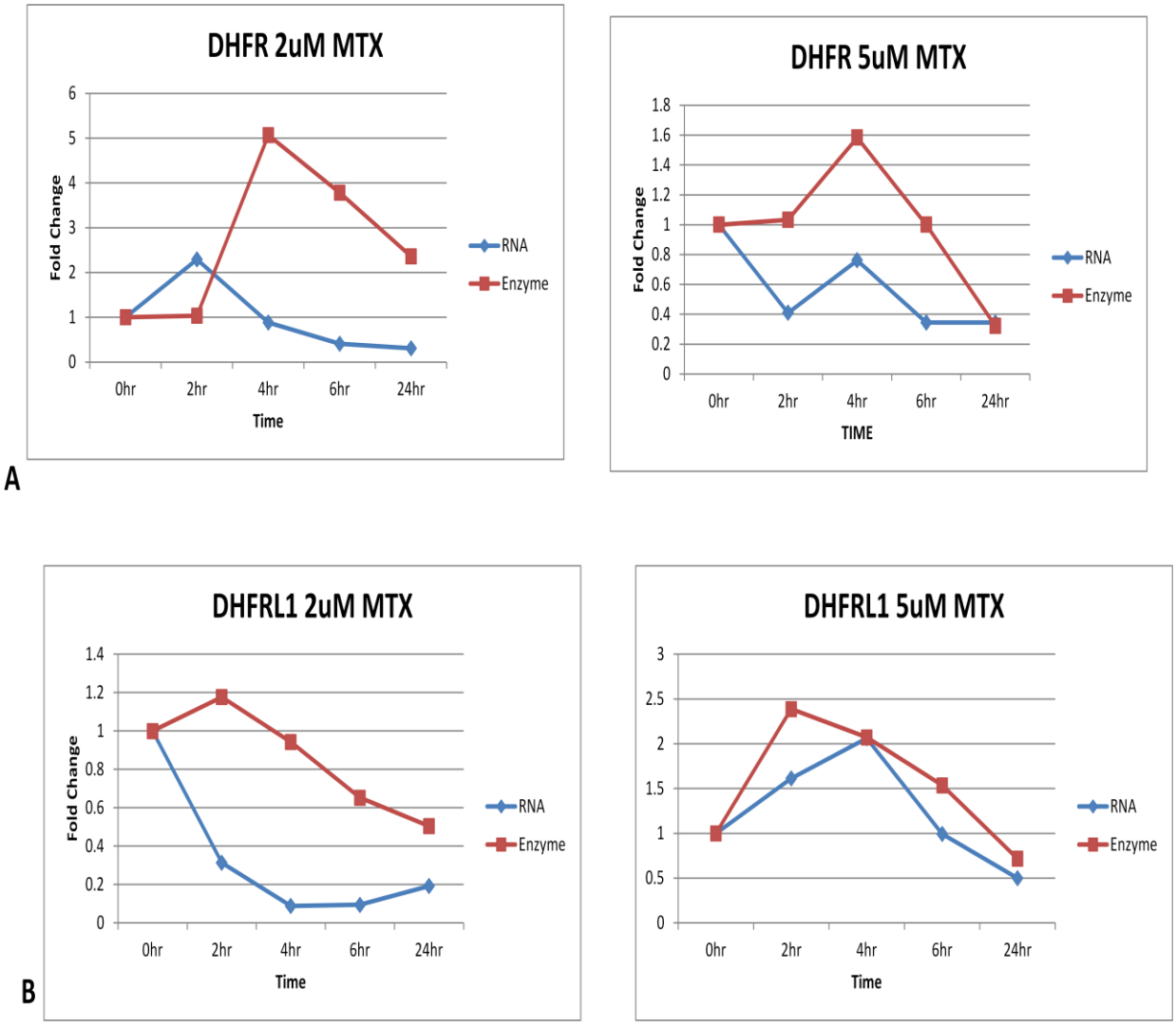

Figure 6.8 Comparison of Enzyme activity and RNA expression after MTX treatment. **(A)** DHFR shows an increase in enzyme activity 4 hours after treatment with either 2µM or 5µM MTX. Although there also appears to be a slight increase in RNA levels after treatment the increase in expression is much less than the increase in enzyme activity. **(B)** DHFRL1 shows a relatively small increase in enzyme activity 2 hours after treatment with 2µM MTX and RNA levels show a decrease after treatment at this concentration. There is an increase in enzyme activity 2 hours after treatment with 5µM MTX while RNA levels also show a slight increase in expression after treatment again the increase in expression is lower compared to the increase in enzyme activity. These results would indicate that while some of the increased enzyme activity may be due to a slight increase in expression levels it is most likely that the majority of the enzyme activity comes from protein released from the RNA: protein binding complex.

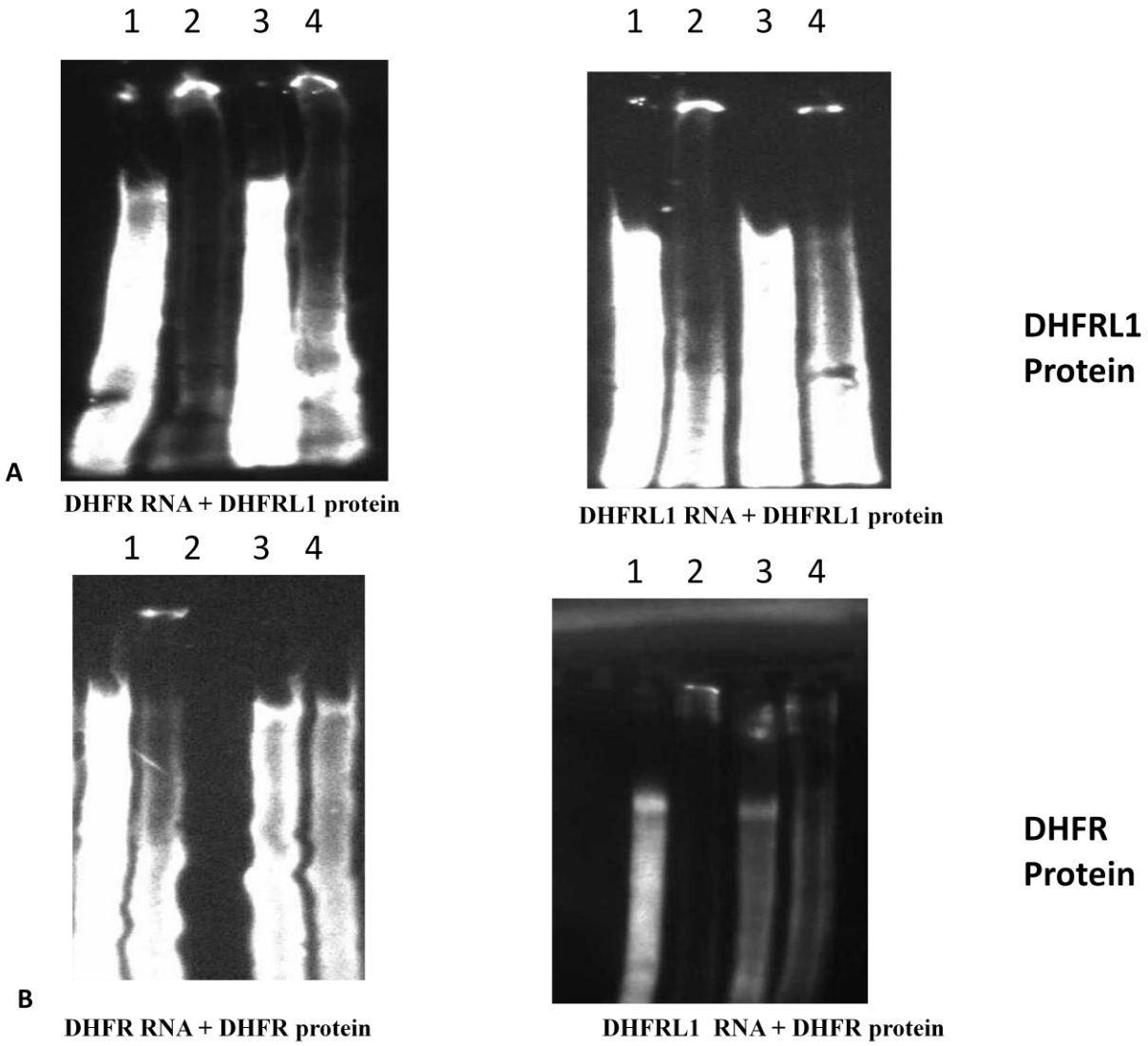

Figure 6.9 REMSA experiments with 2µM MTX. REMSA experiments were carried out with both DHFR and DHFRL1 RNA bound to either DHFRL1 protein **(A)** or DHFR protein **(B)**. For each blot the lanes 1-4 contained; lane 1 labelled RNA only, lane 2 labelled RNA bound to protein, lane 3 labelled RNA, protein and unlabelled RNA and lane 4 labelled RNA, protein and 2µM MTX. DHFRL1 protein binding to either DHFR or DHFRL1 RNA was unaffected by 2µM MTX. DHFR protein binding to both DHFR and DHFRL1 RNA however was disrupted by 2µM MTX.

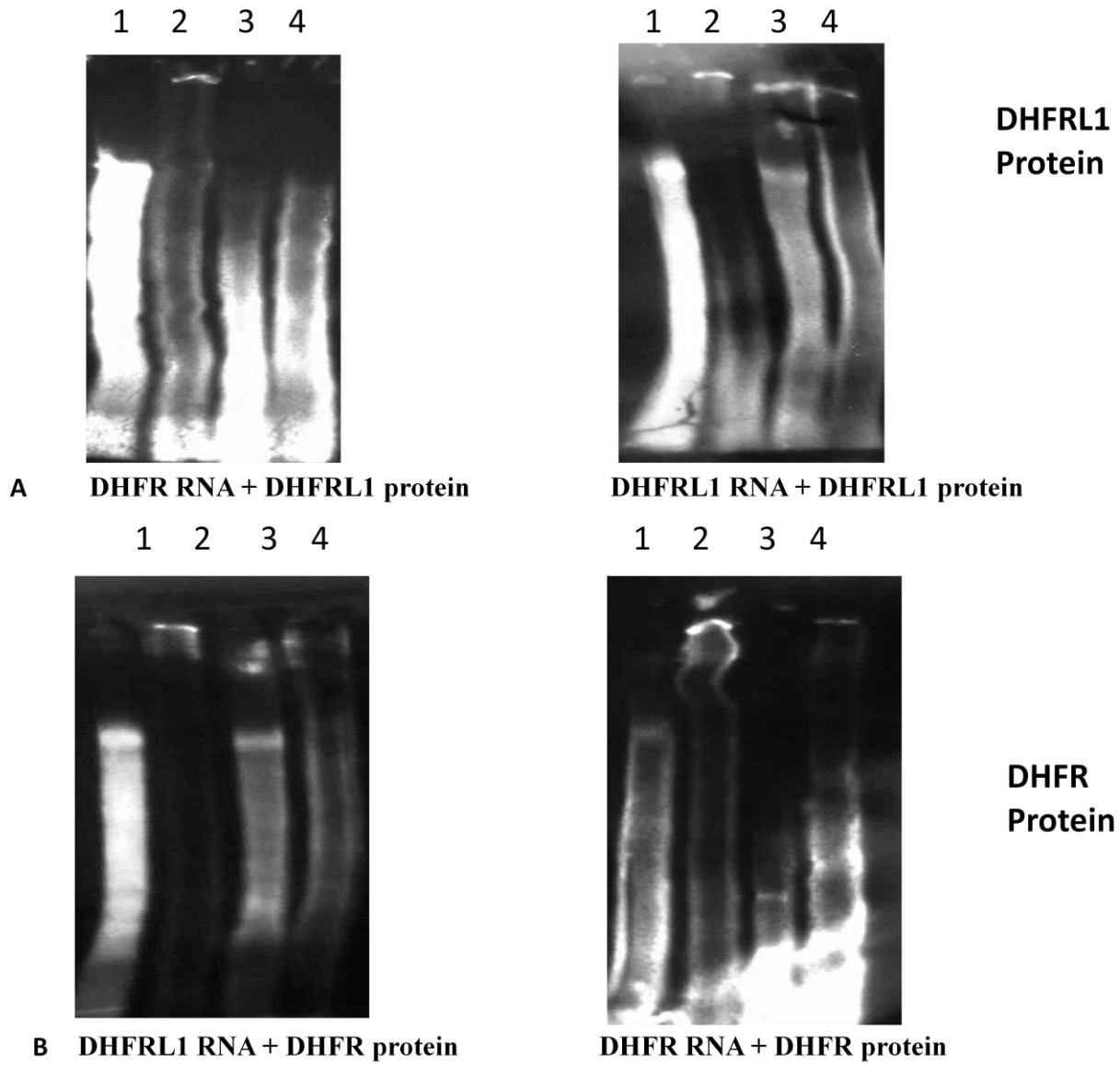

Figure 6.10 REMSA experiments with 5µM MTX. REMSA experiments were carried out with both DHFR and DHFRL1 RNA bound to either DHFRL1 protein (A) or DHFR protein (B). For each blot the lanes 1-4 contained; lane 1 labelled RNA only, lane 2 labelled RNA bound to protein, lane 3 labelled RNA, protein and unlabelled RNA and lane 4 labelled RNA, protein and 2µM MTX. DHFRL1 protein binding to either DHFR or DHFRL1 RNA was disrupted by 5µM MTX. DHFR protein binding to both DHFR and DHFRL1 RNA was also disrupted by 5µM MTX.

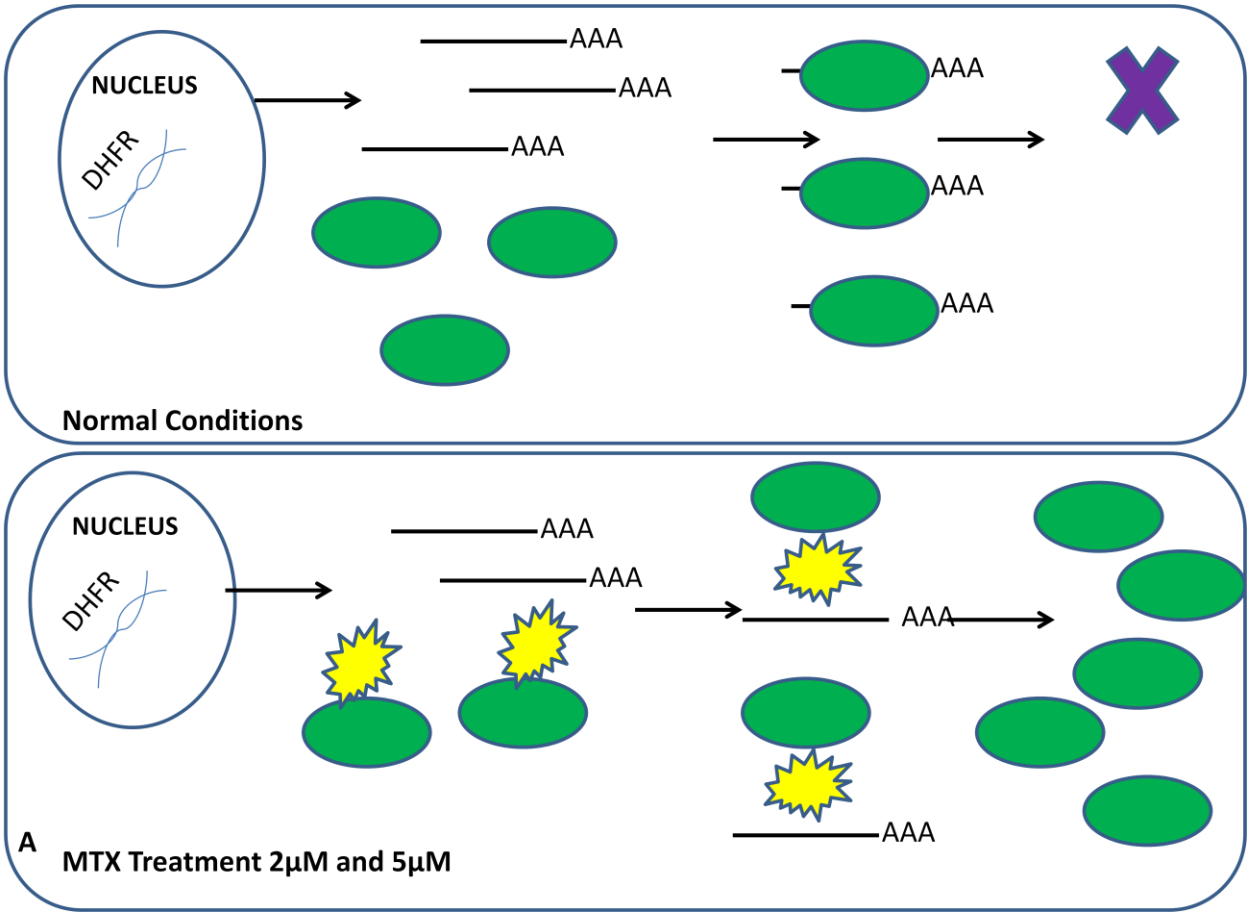

Figure 6.11 Initial response of DHFR to MTX treatment. Under normal conditions in the cell DHFR mRNA undergoes translation into protein. When less protein is required DHFR can suppress translation by binding to mRNA molecules and preventing them from undergoing translation. When MTX was introduced into the cell at either 2µM or 5µM it disrupts the binding complex which results in an initial increase in enzyme activity as a result of increase protein levels.

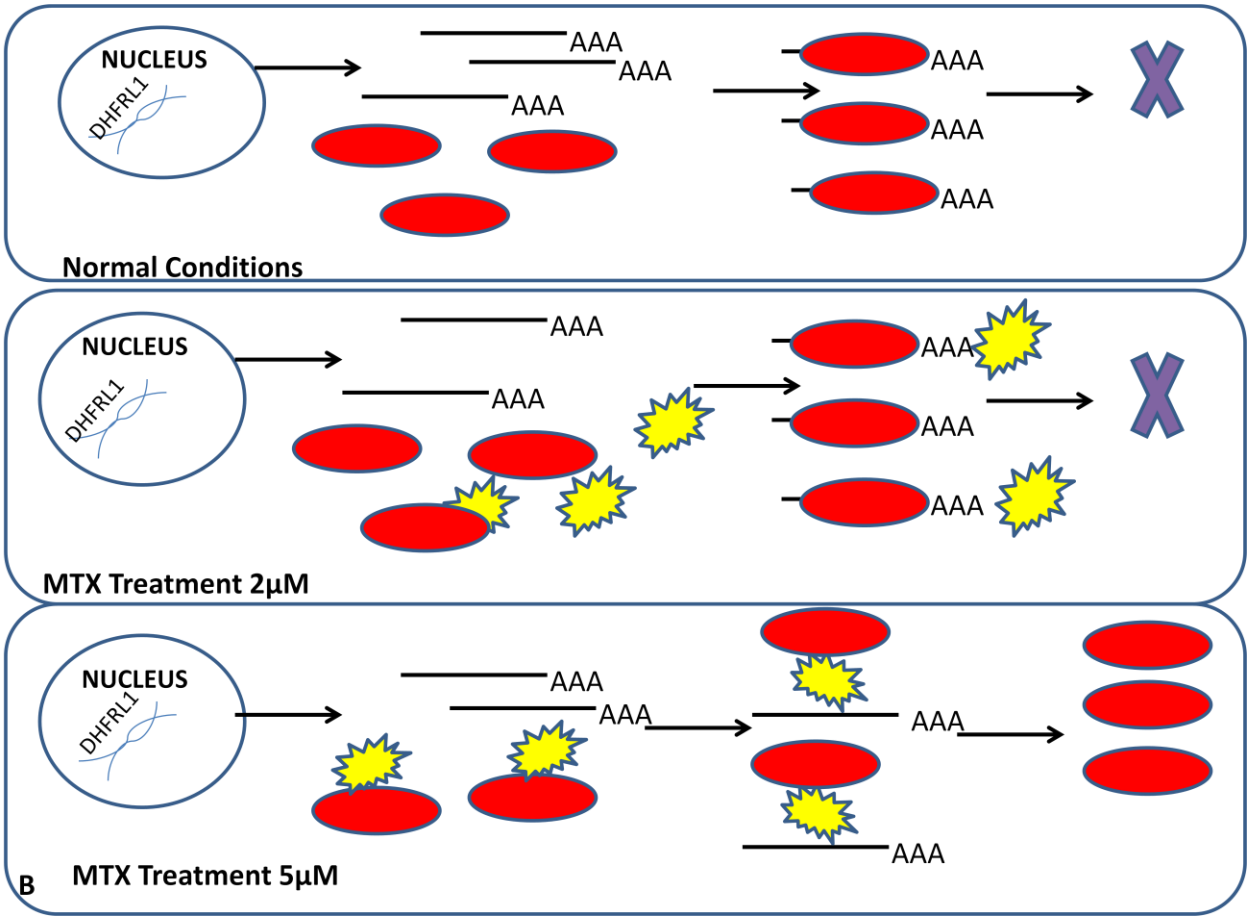

Figure 6.12 Initial response of DHFRL1 to MTX treatment. Under normal conditions in the cell DHFRL1 can also suppress translation by binding to mRNA molecules and preventing them from undergoing translation. When MTX was introduced into the cell at 2µM concentration this binding complex remained undisturbed however when MTX concentration was increased to 5µM disruption of the binding complex occurred which resulted in an initial increase in enzyme activity and increase in protein levels.

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Chapter 8

General Discussion

8.1 Discussion

Folates act as donors and acceptors of one carbon units in a variety of reactions known as one-carbon metabolism. These reactions include but are not limited to thymidylate and *de novo* purine biosynthesis and methyl group catabolism.[172] Folate metabolism is essential to the survival of the cell and any impairment to the pathway can have severe consequences for the health of the individual. As discussed previously defects in the folate mediated one – carbon metabolism pathway can increase the risk of a wide range of diseases including; NTDs, CVD, cancer, Alzheimer's, Parkinson disease and also depression. While low folate levels in the diet contribute to the increase risk of developing these diseases, the underlying mechanism behind the disease is usually a combination of environmental factors, such as low folate status, and gene polymorphisms in one or more of the essential folate enzymes.

In order for cells to retain folates they must first be converted to polyglutamate derivatives by the enzyme folypoly-γ-glutamate synthase (FPGS). A number of studies have confirmed that the polyglutamate forms are the more effective substrate for the enzymes of one-carbon metabolism.[173] Folate polyglutamates have been identified in all sub-cellular organelles,[174] however the majority of folate mediated one-carbon metabolism is compartmentalized between the cytoplasm and the mitochondria, with the nuclei containing only low levels of folate.[175]

Mitochondria contain almost 40% of total cellular folate however the folate polyglutamates found in the mitochondria differ from those found in the cytoplasm.[176] In the cytoplasm the majority of folate is found in the form of methyl-tetrahydrofolate (45%) whereas in the mitochondria 44% of total folate is found in the form of formyl-tetrahydrofolate.[176] Despite this folate metabolism that occurs in the mitochondria is not distinct from that which occurs in the cytoplasm rather both pathways are inter-dependant, with one-carbon metabolism in the mitochondria producing formate which is then transported to the cytoplasm for use in cytoplasmic one-carbon metabolism.[175]

Folate mediated one – carbon metabolism is an extensively studied pathway due to its essential role within the cell, however our knowledge and understanding of this pathway is constantly evolving. Previously it was believed that all one carbon reactions were carried out in the cytoplasm and any products required by either the mitochondria or the nucleus was transported

to the organelle as required. It is now understood that this is not always the case and that some of the reactions are carried out within the organelle itself. For example *de novo* thymidylate synthesis is essential for the repair and replication of DNA and was previously thought to be produced solely in the cytoplasm and transported to the nucleus and the mitochondria. It has recently been shown by the Stover lab in Cornell University that the *de novo* thymidylate synthesis pathway is present in both the nucleus[177] and the mitochondria[105], so some if not all thymidylate is actually produced in house in these organelles.

Other recent advances in the study of folate metabolism include the discovery of new folate enzymes, particularly in the mitochondria which is increasingly coming under stronger scrutiny as more knowledge is acquired about folate metabolism in this organelle. For example the reactions carried out in the cytoplasm by the enzyme MTHFD1, which through a number of reactions converts THF to 5, 10 methylene (see Figure 8.1), are carried out in the mitochondria by the homologous enzymes MTHFD1L,[178] MTHFD2 and MTHFD2L. [179] Figure 8.1 gives an overview of the compartmentalisation of folate mediated one - carbon metabolism.

Folate mediated one-carbon metabolism is a complex process involving numerous different enzymes and occurring in multiple locations in the cell. The question arises - where does DHFRL1 fit into the overall folate metabolism complex? To answer this question it is necessary to first examine the role of DHFR in folate metabolism.

One of the initial reactions of folate metabolism is to reduce dihydrofolate into tetrahydrofolate. This key reaction is catalyzed by DHFR in the presence of NADPH. DHFR is also the only known enzyme to reduce folic acid the synthetic form of folate.

The *DHFR* gene family consists of one functional gene located on chromosome 5 [70] one retrogene (*DHFRL1*), formerly annotated as a pseudogene, located on chromosome 3, (the re-classification of DHFRL1 is based on work presented in this thesis as well as work published by McEntee *et al*[150] and Anderson *et al* [105]) and three processed pseudogenes scattered throughout the genome, (Table 8.1 lists the members and chromosomal locations of the *DHFR* gene family). Pseudogenes are generally viewed as genomic fossils that have a high sequence similarity to the parent gene but are essentially non-functional. With increasing evidence to suggest that

pseudogenes do have a role to play within the cell, primarily in the regulation of their functional counterpart, the traditional view of pseudogenes is being challenged.

The pseudogenes of *DHFR* have many of the characteristics needed to classify them as processed pseudogenes. All lack introns indicating that they were generated from an mRNA intermediate. The *DHFRP1* pseudogene is polymorphic in the human population and contains an ORF identical to that of *DHFR* however it does not appear to have any promoter activity. *DHFRP2* contains several stop codons with the ORF which would result in a truncate protein if expressed and *DHFRP3* contains only the 3' half of the *DHFR* coding sequence again making expression most unlikely.

The work presented in this thesis concerns the fourth annotated pseudogene *DHFRP4* or as it is more commonly known *DHFRL1* which is located on chromosome 3 and like the other pseudogenes does not have any introns, a classical characteristic of a processed pseudogene however recent evidence reveals that *DHFRL1* is in fact a retrogene and is functional within the cell. *DHFRL1* has a 92% sequence homology to the functional *DHFR* gene but what distinguishes *DHFRL1* from the other pseudogenes is not only an ORF without in-frame stop-codons and promoter activity but also the necessary signals for translation. Another important distinction between *DHFRL1* and its sister pseudogenes is that two transcripts for the *DHFRL1* gene are annotated in the Ensembl database (www.ensemble.org). The presence of a *DHFRL1* transcript was confirmed in a number of human cell lines, both cancerous and non-cancerous, by RT-qPCR.[150] Enzyme kinetic analysis and localization studies, described in this thesis, subsequently revealed that DHFRL1 protein does have dihydrofolate reductase activity, with a much lower affinity for the substrate, and the protein has a strong presence in the mitochondria.

The majority of experiments detailed in this thesis used recombinant DHFRL1 protein produced in an *E-coli* host system. This approach has both advantages and disadvantages. One of the main advantages to using this system is that large amounts of protein produced and purified with relative ease in a short space of time, with scale up reactions easily accomplished. The use of *E-coli* in the production of recombinant protein is a widely used molecular tool with a number of mutant strains, such as BL21, specifically developed for enhanced protein expression.

There are two major disadvantages to using this host system; the first being the misfolding of protein and the formation of insoluble inclusion bodies which makes purification of the protein difficult. This obstacle can often be overcome by reducing the temperature of growth, optimizing the concentration of the inducing agent, switching the fusion tag from one terminal to the other e.g. from the N terminal to the C terminal or choosing a different fusion tag such as GST or maltose binding protein (MBP). The second disadvantage to this system is the limited post-translational modifications which occur in *E-coli* and which often differ from those found in eukaryotic cells. *E-coli* cells do not facilitate any form of glycosylation, amidation or hydroxylation, which are just three mechanisms of post translational modifications commonly found in eukaryotic cells.[180] Recombinant protein can be produced in mammalian cells which would allow the protein to undergo post translational modifications, however using mammalian cells as a host is often time consuming, expensive and is difficult to produce large amounts of proteins as scale up reactions can be difficult.

Although there are acknowledged limitations to using recombinant proteins to study functional activity, they do provide initial insights which may prove valuable in future studies concerning the endogenous protein.

The identification of a second dihydrofolate reductase enzyme in humans is of major significance and this work, with the majority of experiments outlined in this report, was published in the Proceedings of the National Academy of Science USA (PNAS) [150] as a back to back publication with Dr. Patrick Stover's lab in Cornell University New York. Research carried out by the Stover group focused on the role of DHFRL1 in the mitochondria.

In the cytoplasm DHFR is involved in one of three folate mediated one-carbon metabolism pathways, namely de novo dTMP biosynthesis. The other two pathways are de novo purine biosynthesis and homocysteine re-methylation.[181] The synthesis of de novo dTMP occurs through the conversion of dUMP to dTMP and is catalyzed by three folate enzymes serine hydroxymethyltransferase (SHMT), thymidylate synthase (TS) and DHFR.[120] This reaction also takes place in the nucleus with the relevant enzymes including DHFR being sumoylated and targeted to the nucleus during the S phase of the cell cycle. [177]

The synthesis of dTMP is necessary for the replication of all DNA, nuclear and mitochondrial, and until recently it had been believed that dTMP biosynthesis in the cytoplasm supported both. However the Stover group [105] identified a de novo thymidylate biosynthesis pathway in mitochondria with DHFRL1 taking the place of DHFR within the reaction. In this study when DHFRL1 expression was knocked down by siRNA, reductase activity was significantly reduced in the mitochondria but was unaffected in the cytoplasm confirming that DHFR is not present in the mitochondria but is "replaced" by DHFRL1. Figure 8.2 shows the role of DHFRL1 in folate metabolism in the mitochondria.

The mechanism to facilitate transport to the mitochondria is unclear given that DHFRL1 has no obvious mitochondrial targeting sequence. Mitochondrial targeting sequences (MTS) do not appear to share a consensus primary sequence rather they share similar overall characteristics. Most MTS lack acidic residues and are rich in the positively charged amino acids arginine and lysine and the hydroxylated amino acids serine and threonine.[182] A secondary feature common among MTS is the formation of an amphiphilic α helices found on the surface of proteins.[183] Hurt and Schatz found that amino acids 1-85 on mouse DHFR had the potential to be a MTS; however it was inactive within the folded protein.[184] These amino acids are highly conserved within DHFRL1 (see Figure 8.3) and therefore the localisation of DHFRL1 to the mitochondria may be related to the folding of the protein after translation revealing the presence of amino acids that facilitate its import.[185] The site directed mutagenesis experiments would seem to indicate that the three amino acids FLL at the N terminal of DHFRL1 protein are important for mitochondrial localisation as when they were changed to the amino acids found at the same position in DHFR protein, VGS, localisation to the mitochondria was no longer observed. Although the exact mechanism may still be unclear, the localization of DHFRL1 to the mitochondria does however highlight the importance of mitochondrial folate metabolism and adds another enzyme to the growing list of mitochondrial specific folate enzymes.

Although the evidence clearly indicates that DHFRL1 is present in the mitochondria it may not be exclusive to that organelle, as a presence in the cytoplasm or nucleus cannot be ruled out. Determining the exact location of DHFRL1 protein, particularly during the cell cycle, will be important in fully characterizing DHFRL1 at a molecular level, which may offer us further insights, not only into folate metabolism, but also into DHFR and particularly how these two

highly similar proteins interact with each other. For instance what role if any does DHFRL1 play in regulating translation of DHFR? RNA binding experiments detailed in McEntee *et al* [150] showed that DHFRL1 protein can bind DHFR mRNA and DHFR protein can bind DHFRL1 mRNA. This binding is significant as it indicates that the auto-regulation of DHFR may need to be reassessed to include DHFRL1.

The post-translational modification of DHFR by the protein SUMO-1 facilitates the localisation of DHFR to the nucleus during S phase of the cell cycle. The binding motif for sumoylation is conserved within DHFRL1 and *in vitro* experiments on recombinant DHFRL1 protein indicate that this motif is active. During the S phase of the cell cycle DNA synthesis is at its peak and as a result higher concentrations of DHFR protein are required for sumoylation to the nucleus, resulting in an increase in *DHFR* gene expression during this phase of the cell cycle. Expression of one of the DHFRL1 transcripts, the T2 transcript, is also increased during the S phase of the cell cycle indicating that more DHFRL1 protein is also required during this phase of the cell cycle. Although mitochondrial DNA replication is known to occur at all stages of the cell cycle and is not dependent on nuclear DNA synthesis, there is evidence to suggest that it may be increased during the S phase of the cell cycle. If this is the case then due to its role in mitochondrial *de novo* thymidylate synthesis it is logical that an increase in DHFRL1 protein would be required. However if DHFRL1 also undergoes sumoylation during this phase of the cell cycle is it also possible that DHFRL1 goes to the nucleus? If proven correct this raises the question does DHFRL1 have another function within the cell which would require its presence in both the mitochondria and nucleus, as DHFR in the nucleus functions in the nuclear *de novo* thymidylate synthesis pathway? This would require further investigation and proof that DHFRL1 does actually go to the nucleus.

The discovery of a second dihydrofolate reductase enzyme is also relevant for developing therapeutic strategies. DHFR is the central target for a number of anti-folate drug therapies including MTX. MTX competes with the substrate dihydrofolate to bind to the enzyme's active site preventing binding of the substrate which in turn depletes the pool of available tetrahydrofolate directly affecting cell proliferation. Characterization of DHFRL1 enzyme revealed a lower binding affinity for the substrate and unsurprisingly also a lower binding affinity for MTX. There is considerable evidence showing that MTX disrupts the DHFR mRNA:

protein binding complex leading to an initial increase in DHFR activity after exposure to the drug. Due to the diminished response of DHFRL1 to MTX a higher concentration of the drug is required to disrupt the DHFRL1 protein: mRNA (either DHFRL1 or DHFR) binding complex. The reduced ability of MTX to disrupt DHFRL1 protein: RNA binding complex may diminish the therapeutic effectiveness of the drug. If all the MTX administered is bound to available DHFR then the pools of available tetrahydrofolate will be depleted which may trigger the release of the DHFRL1 protein bound to DHFR mRNA allowing the mRNA to be translated increasing concentration of free DHFR protein and thus replenishing the pools of available tetrahydrofolate making the drug ineffective.

A further obstacle of using MTX to target DHFRL1 is the presence of DHFRL1 in the mitochondria. MTX is unable to enter the mitochondria and therefore would not have access to the protein present there. Although there has been developments in targeting drugs to the mitochondria using conjugation techniques, due to DHFRL1 low binding affinity to MTX this drug may not be the most effective at targeting DHFRL1. As DHFR has been a target for anti-folate drug therapies since the 1950's it is possible that one or more of the other DHFR targeting drugs will also be effective against DHFRL1. It is possible that a dual drug approach targeting both DHFR in the cytoplasm and DHFRL1 in the mitochondria may lead to a more improved clinical outcome.

The presence of a second functional dihydrofolate reductase enzyme in humans, particularly one that has a presence in the mitochondria, raises the question is there a second dihydrofolate reductase enzyme in other species? This is a relevant question on both an evolutionary level and also on a more practical laboratory level where animal are used as model organisms. Bioinformatic analysis has shown that the event from which *DHFRL1* evolved was primate specific, so *DHFRL1* does not have an ortholog in species below primates. This is unsurprising given that recent data from the ENCODE project reveals a high level of retro transposition in primates with approximately 80% of human processed pseudogenes being primate specific. The fact that *DHFRL1* is primate specific also raises a number of questions, particularly regarding the role of DHFRL1 in *de novo* synthesis pathway in the mitochondria. In non-primate species is this role in the mitochondria carried out by DHFR or is there a second enzyme present? If DHFR in other species functions in *de novo* thymidylate synthesis in the mitochondria how or why did

primate DHFR loose this function? Or alternatively if there is a second enzyme present in species other than primates why was this enzyme discarded in favour of DHFRL1 in primates?

Before any or all of these questions can be answered a second dihydrofolate reductase enzyme must be shown to be present in other mammals. A bioinformatic search for a second potential dihydrofolate reductase enzyme in rodents produced a potential candidate in both mouse and rat. The candidate sequence named *DHFRLS* (dihydrofolate reductase like sequence) appeared to be expressed in mouse tissues, although *DHFRLS* had lower expression levels compared to *DHFR*. *DHFRLS* also had very low levels of expression in rat tissue samples compared to *DHFR*. Subsequent cloning and localisation experiments did not reveal a presence of this DHFRLS in the mitochondria in either mouse or rat cells, which would seem to indicate that if this candidate is actually a second dihydrofolate reductase enzyme in rodents it is unlikely to be involved in *de novo* thymidylate synthesis in the mitochondria. However mitochondria isolated from mouse and rat tissue samples showed some dihydrofolate reductase activity, so the question still remains does this activity come from DHFR or is there a second enzyme present similar to DHFRL1 in humans?

Although interesting from an evolutionary point of view confirmation of the presence or absence of a second enzyme in other mammals is also important from a practical point of view. Model organisms, including mouse and rat, are often used to study complex pathways, including folate metabolism, especially their role in disease. While no model organism can mimic the conditions of the human body exactly using the correct model organism is an essential parameter when designing an experiment. For example any future experiments in further characterizing DHFRL1, particularly its role in health and disease, which would require a model organism such as a knockout mouse would need to ensure that a second enzyme with similar function to DHFRL1 was present in that organism. Alternatively the study of DHFRL1 may be limited to cell culture models where the gene can be knocked down using siRNA technology or alternatively over expressed to determine how levels of DHFRL1 affect folate metabolism in the cell.

DHFR has previously been thought to be the only enzyme capable of reducing dihydrofolate into tetrahydrofolate in humans, a reaction essential for cell proliferation. The work presented here refutes that assumption and shows that DHFRL1 is not as previously thought a pseudogene of

DHFR but rather a retrogene that is expressed and functional, performing a vital role in the *de novo* thymidylate synthesis pathway in the mitochondria.

8.2 Future Work

Until recently it was assumed that humans had only one enzyme capable of reducing dihydrofolate, and the DHFR gene family was believed to consist of one functional gene, on chromosome 5, and four pseudogenes scattered across the genome. Present knowledge indicates that this assumption is incorrect and that a second enzyme is coded for by *DHFRL1* on chromosome 3, an annoted pseudogene which is in fact a retrogene. Table 8.1 displays our current knowledge of the dihydrofolate reductase gene family; their chromosomal locations and their function within the cell.

As the discovery that *DHFRL1* encodes a second enzyme is only recent, our knowledge of this new folate enzyme is not yet complete. Our present knowledge of DHFRL1 tells us that it is a functional dihydrofolate reductase enzyme, albeit with a reduced affinity for the substrate dihydrofolic acid, which localizes to the mitochondria where it is understood to be involved in *de novo* thymidylate synthesis. DHFRL1 can also act as an RNA binding protein and can bind and suppress translation of both its own mRNA and also that of DHFR; DHFR protein has also been shown to bind to DHFRL1 mRNA as well as its own to suppress translation. DHFRL1 contains the consensus motif required for the post-translational modification by the protein SUMO-1. *In vitro* experiments on recombinant DHFRL1 protein show that his motif is active and that DHFRL1 may undergo sumoylation *in vivo*. For DHFR sumoylation facilitates its transport to the nucleus during the S phase of the cell cycle, it is possible it may perform a similar role for DHFRL1. As a therapeutic target DHFRL1 shows a reduced affinity for the anti-folate MTX which is perhaps unsurprising given that MTX competes with the substrate to bind to the enzyme and DHFRL1 already has a lower affinity for the substrate compared to DHFR.

Future work on this new folate enzyme may include:

- **Further investigation into the localisation of DHFRL1.**

As previously discussed in detail DHFRL1 has a strong presence in the mitochondria. The exact mechanism of this localisation is unclear given the lack of a mitochondrial targeting sequence; however it would appear that the FLL amino acids at the N terminal of the protein contribute to the localisation in some way. The exact nature of how these amino acids, if all three are required or just some of them, facilitate mitochondrial localisation is unknown. Do these amino acids change the folding of the protein revealing the presence of a mitochondrial targeting sequence on the surface which allows targeting to the mitochondria? Or are they involved in signalling to another protein which facilitates transport into the mitochondria? Further investigation is required to elucidate the exact mechanism by which DHFRL1 is imported into the mitochondria.

Aside from its presence in the mitochondria it is also important to establish if DHFRL1 has a presence in the cytoplasm or nucleus. Confocal experiments carried out in this thesis stained only for the mitochondria and while Western blot results did not indicate a presence in the cytoplasm it may be possible that a small percentage of the protein is found in the cytoplasm which was undetected by the Western blot.

The data produced in this thesis also indicates that the sumoylation motif in DHFRL1 is active and that DHFRL1 may be post-translationally modified by the SUMO-1 protein. The sumoylation process has been known to facilitate the transport of proteins to other organelles including the nucleus. Further experimental evidence is required to determine if DHFRL1 undergoes sumoylation in a cell culture model and if this transports the protein to the nucleus similar to DHFR.

Confirmation of the presence or absence of DHFRL1 in either the cytoplasm or nucleus at certain stages of the cell cycle may also help to determine why an increase in DHFRL1 expression is observed during the S phase of the cell cycle at the RNA level.

- **DHFRL1 as a therapeutic target**

DHFR is the target of a number of anti-folate drug therapies used in the treatment of a broad spectrum of diseases including cancer, malaria and rheumatoid arthritis. As it was unknown until now that that a second enzyme existed the response of DHFRL1 to any of these drugs is unknown. Determining the exact localisation of DHFRL1 will also be crucial in developing therapeutic strategies as not all anti-folates e.g. MTX can actually enter the mitochondria. Therefore developing DHFRL1 as a potential therapeutic target may lead to two avenues investigation; 1) finding an anti-folate drug which effectively targets DHFRL1 and 2) targeting that drug to the organelle/organelles which DHFRL1 has a presence.

- **The regulation of DHFRL1 activity**

DHFR activity is tightly regulated at every level from transcription through to post – translation. In this thesis data is presented which indicates that on a translational level DHFRL1 is controlled by an RNA binding mechanism similar to the regulation of DHFR translation, and that DHFRL1 mRNA translation can be suppressed by the binding of either DHFRL1 or DHFR protein. DHFRL1 may also have the same post – translational modification mechanism as DHFR with evidence suggesting that DHFRL1 may also undergo sumoylation.

The transcriptional regulation of DHFRL1 is as yet unexplored. DHFR is regulated at the transcriptional level by two separate mechanisms; 1) the binding of transcription factors Sp2 and E2F to the major promoter and 2) through the interference of an ncRNA coded by the minor promoter which binds to the major promoter causing the disassociation of the pre-initiation complex. As regulation of DHFRL1 at the translational and post-translational level mirror that of DHFR it is possible transcriptional regulation may also be similar to that of DHFR. It may also be possible that DHFRL1 activity is regulated on some level by the second transcript, T1, which differs from the T2 transcript by having a longer 5'UTR. RT-qPCR expression analysis indicates that while the T1 transcript is expressed it is at much lower levels compared to the T2 transcript and also levels of this transcript remain relatively unchanged during the different phases of the cell cycle. Further investigation is required to determine what function the DHFRL1 T1 transcript has in the cell.

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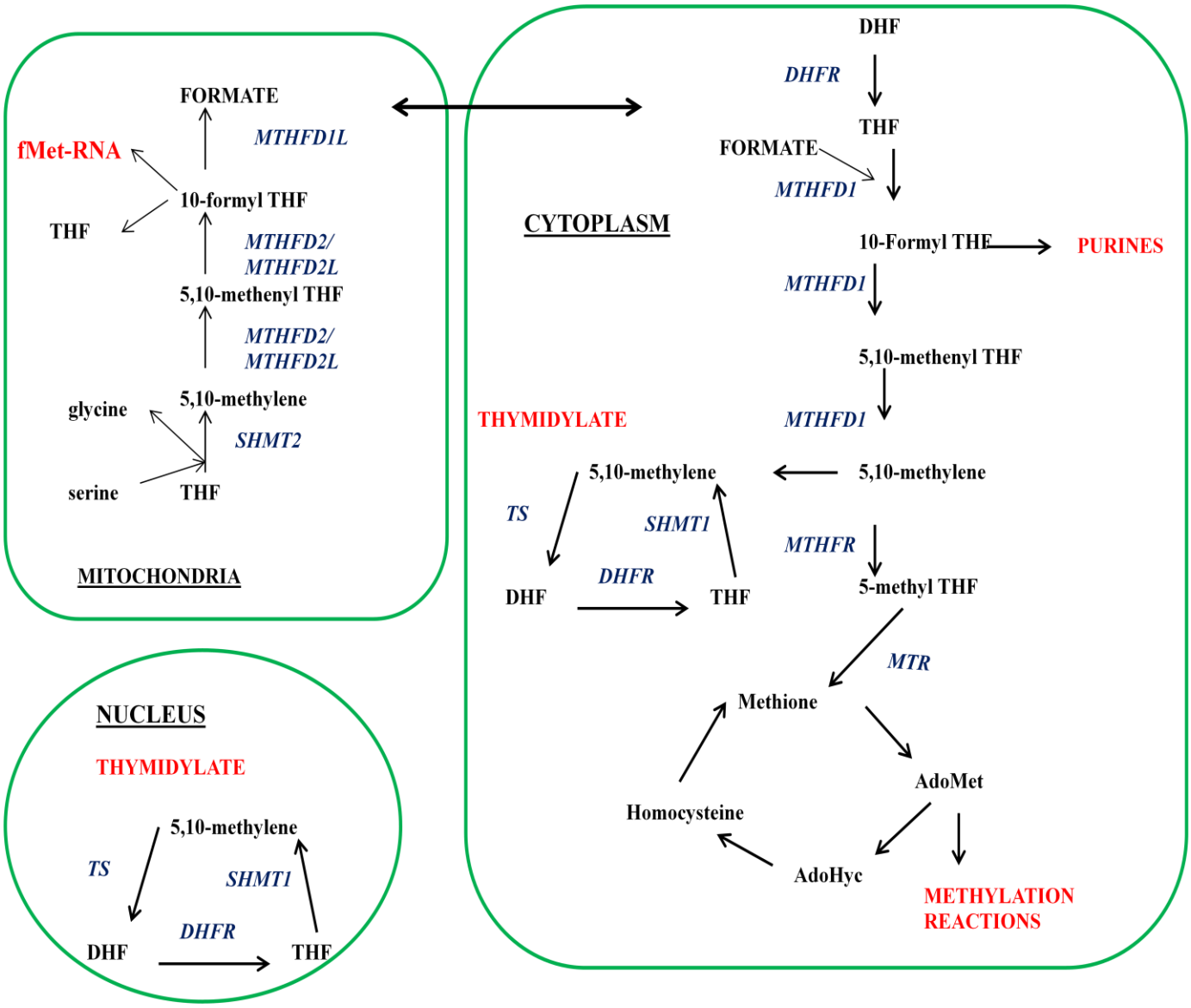


Figure 8.1 Compartmentalisation of one-carbon metabolism. Image adapted from Tibbetts AS and Appling DR. (2010). *Annu. Rev. Nutr.* **30:**57-81. Folate mediated one carbon metabolism is compartmentalised in the cytoplasm, mitochondria and to a lesser extent the nucleus. The pathways are not distinct from each other but are interdependent; folate metabolism in the mitochondria produces formate which is used in cytoplasmic folate metabolism while the enzymes required for thymidylate synthesis in the nucleus are transported from the cytoplasm facilitated by sumoylation.

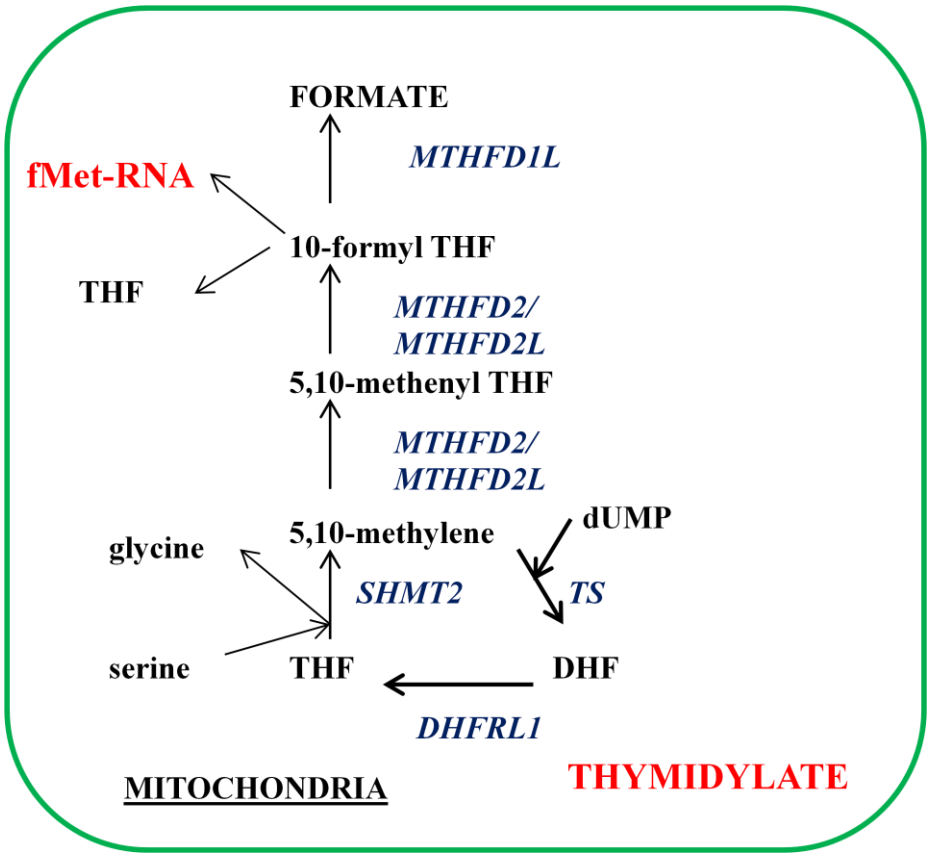


Figure 8.2 Role of DHFRL1 in the mitochondria. Image adapted from Tibbetts AS and Appling DR. (2010). *Annu. Rev. Nutr.* **30:**57-81. in the cytoplasm and the nucleus *de novo* thymidylate synthesis is carried out by DHFR, TS and SHMT1 and any thymidylate required by the mitochondria was believed to be imported. However recent evidence suggests that *de novo* thymidylate synthesis can and does occur in the mitochondria. In the mitochondrial *de novo* thymidylate synthesis pathway DHFR is replaced by DHFRL1 and SHMT1 is replaced by SHMT2.

```
                1                                                   50

HumanDHFRL1<  MFLLLNCIVA VSQNMGIGKN GDLPRPPLRN EFRYFQRMTT TSSVEGKQNL
MouseDHFR  <  MVRPLNCIVA VSQNMGIGKN GDLPWPPLRN EFKYFQRMTT TSSVEGKQNL
Consensus     MfrlLNCIVA VSQNMGIGKN GDLPrPPLRN EFrYFQRMTT TSSVEGKQNL

                                                   85
HumanDHFRL1<  VIMGRKTWFS IPEKNRPLKD RINLVLSREL KEPPQ
 MouseDHFR  < VIMGRKTWFS IPEKNRPLKD RINIVLSREL KEPPR
Consensus     VIMGRKTWFS IPEKNRPLKD RINiVLSREL KEPPr
```

Figure 8.3 Amino Acid sequence alignment of human DHFRL1 and mouse DHFR. Amino acids 1-85 in mouse DHFR have been shown to contain a mitochondrial targeting sequence which is believed to be inactive due to the folding of the protein. These amino acids are highly conserved in human DHFRL1 with a number of the substitutions in the DHFRL1 protein being arginine residues which have been shown to be present in the majority of mitochondrial targeting sequences.

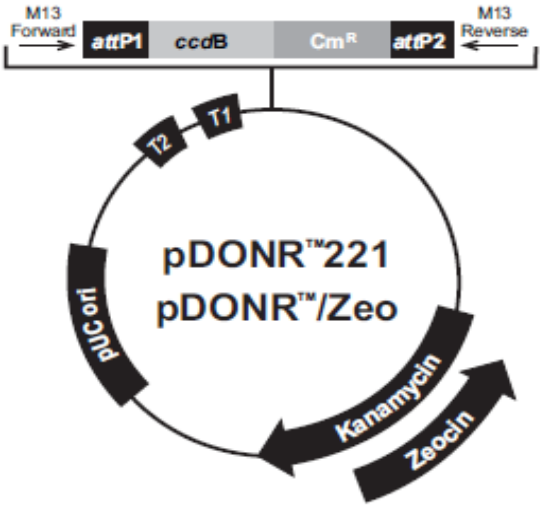
Table 8.1 Current knowledge of the *DHFR* gene family; location and function

Gene Name	Type	Chromosome Location	Function	Protein localization
DHFR	Functional	5 *	Encodes dihydrofolate reductase enzyme	Cytoplasm and nucleus ****
DHFRP1	Processed Pseudogene	18 *	Non-functional	-
DHFRP2	Processed Pseudogene	6 *	Non-functional	-
DHFRP3	Processed Pseudogene	2 **	Non-functional	-
DHFRL1	Retrogene	3 ***	Encodes dihydrofolate reductase enzyme	Mitochondria *****

*Anagnou *et al*., (1984, 1988), ** Shimada *et al*., (1984), *** Maurer *et al*., (1985); Anagnou *et al*., (1984, 1988)

**** Woeller CF *et.al.* (2007), ***** McEntee *et al.,*(2011); Anderson *et al.,*(2011)

Appendices



Comments for:	pDONR™221 4761 nucleotides	pDONR™/Zeo 4291 nucleotides
*rrn*B T2 transcription termination sequence (c):	268-295	268-295
rrnB T1 transcription termination sequence (c):	427-470	427-470
M13 Forward (-20) priming site:	537-552	537-552
*att*P1:	570-801	570-801
*ccd*B gene (c):	1197-1502	1197-1502
Chloramphenicol resistance gene (c):	1825-2505	1847-2506
*att*P2 (c):	2753-2984	2754-2985
M13 Reverse priming site:	3026-3042	3027-3043
Kanamycin resistance gene:	3155-3964	---
EM7 promoter (c):	---	3486-3552
Zeocin resistance gene (c):	---	3111-3485
pUC origin:	4085-4758	3615-4288

(c) = complementary strand

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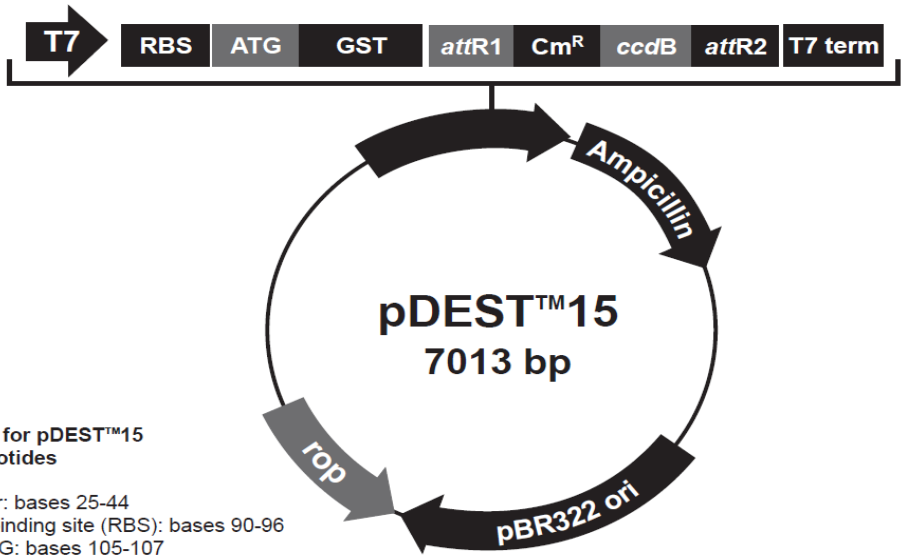
Appendix 2.1 Vector map for pDONR221. Image taken from Invitrogen. Available at http://tools.invitrogen.com/content/sfs/vectors/pdonr221_pdonrzeo_map.pdf



Comments for pDEST™15
7013 nucleotides

T7 promoter: bases 25-44
Ribosome binding site (RBS): bases 90-96
Initiation ATG: bases 105-107
GST tag: bases 108-776
*att*R1: bases 792-916
Chloramphenicol resistance gene (Cm^R): bases 1025-1684
*ccd*B gene: bases 2026-2331
*att*R2: bases 2372-2496
T7 transcription termination region: bases 2518-2646
bla promoter: bases 3134-3232
Ampicillin (*bla*) resistance gene: bases 3233-4093
pBR322 origin: bases 4238-4911
ROP ORF: bases 5282-5473 (C)

C=complementary strand

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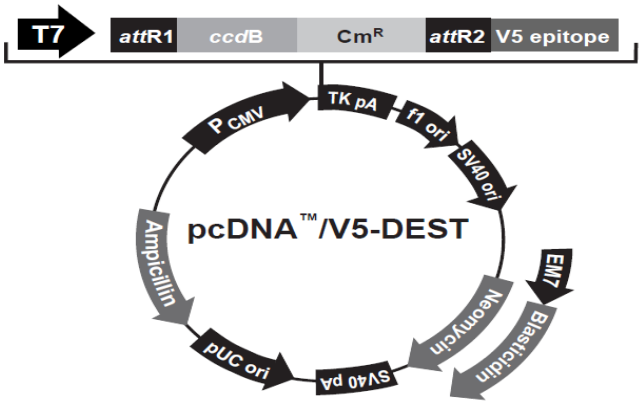
Appendix 2.2 Vector map for pDEST 15. Image taken from Invitrogen. Available at http://tools.invitrogen.com/content/sfs/vectors/pdest15_map.pdf



Comments for:	pcDNA™3.2/V5-DEST 7711 nucleotides	pcDNA™6.2/V5-DEST 7341 nucleotides
CMV promoter:	232-819	232-819
T7 promoter/priming site:	863-882	863-882
*att*R1 site:	911-1035	911-1035
*ccd*B gene (c):	1464-1769	1464-1769
Chloramphenicol resistance gene (c):	2111-2770	2111-2770
*att*R2 site:	3051-3175	3051-3175
V5 epitope:	3201-3242	3201-3242
V5 reverse priming site:	3210-3230	3210-3230
TK polyadenylation signal:	3269-3540	3269-3540
f1 origin:	3576-4004	3576-4004
SV40 early promoter and origin:	4031-4339	4031-4339
Neomycin resistance gene:	4414-5208	---
EM7 promoter:	---	4394-4460
Blasticidin resistance gene:	---	4461-4859
SV40 early polyadenylation signal:	5384-5514	5017-5147
pUC origin (c):	5897-6570	5530-6200
Ampicillin (*bla*) resistance gene (c):	6715-7575	6345-7205
bla promoter (c):	7576-7674	7206-7304

(c) = complementary strand

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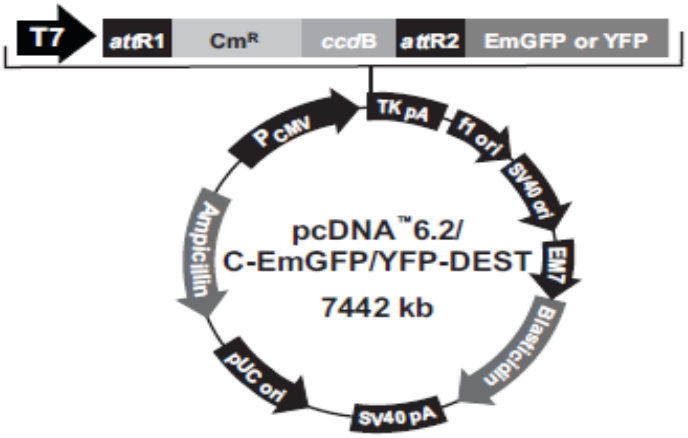
Appendix 2.3 Vector map for pcDNA 3.2/V5. Image taken from Invitrogen. Available at http://tools.invitrogen.com/content/sfs/vectors/pcdnav5dest_map.pdf



Comments for **pcDNA™6.2/C-EmGFP/YFP-DEST**
7442 nucleotides

CMV promoter:	102-689
T7 promoter/priming site:	733-752
attR1 site:	785-909
Chloramphenicol resistance (Cm^R) gene:	1018-1667
ccdB gene:	2019-2324
attR2 site:	2365-2489
EmGFP/YFP:	2503-3222
TK polyA reverse priming site:	3244-3262
TK polyadenylation signal:	3237-3508
f1 origin:	3544-4003
SV40 early promoter and origin:	3999-4307
EM7 promoter:	4362-4428
Blasticidin resistance gene:	4429-4827
SV40 early polyadenylation signal:	4985-5115
pUC origin (c):	5498-6171
Ampicillin (*bla*) resistance gene (c):	6316-7176
bla promoter (c):	7171-7275

(c) = complementary strand

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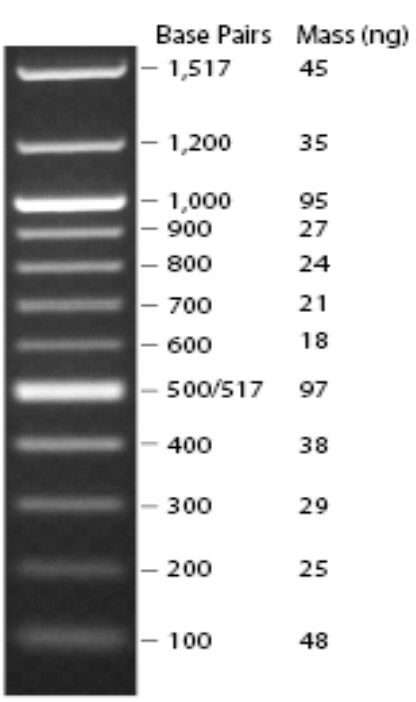
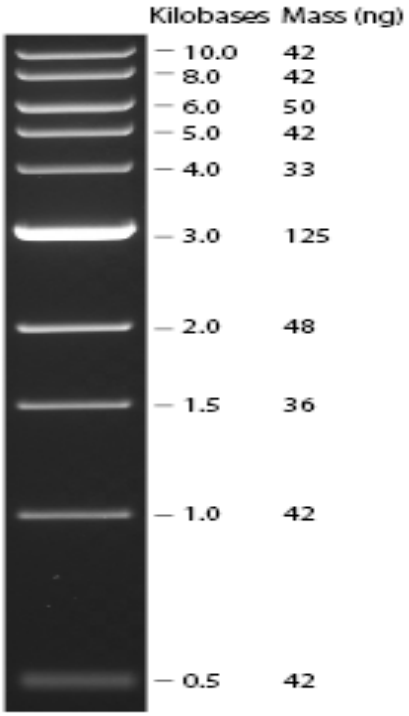
Appendix 2.4 Vector map for pcDNA6.2. Image from Invitrogen. Available at http://tools.invitrogen.com/content/sfs/vectors/pcdna6_2cemgfp_yfp_dest_map.pdf

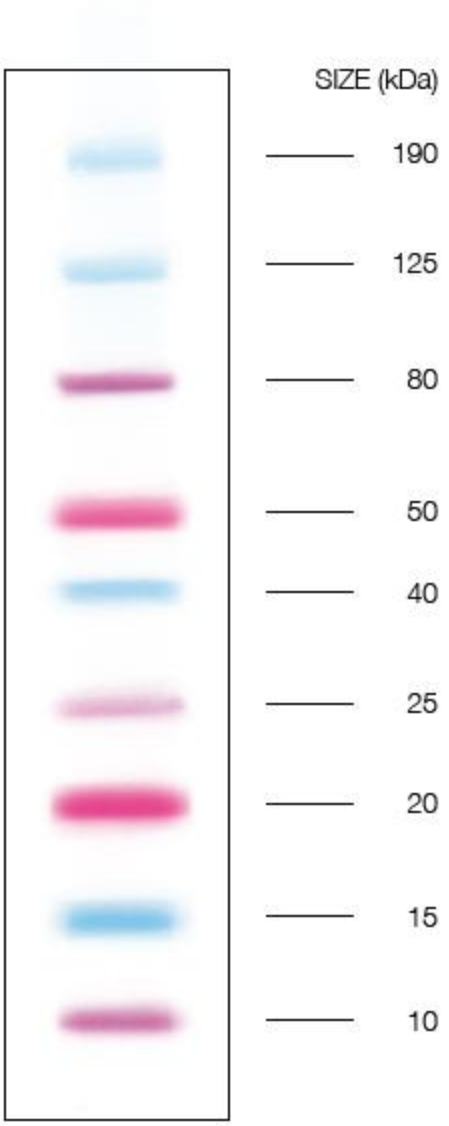


100bp ladder

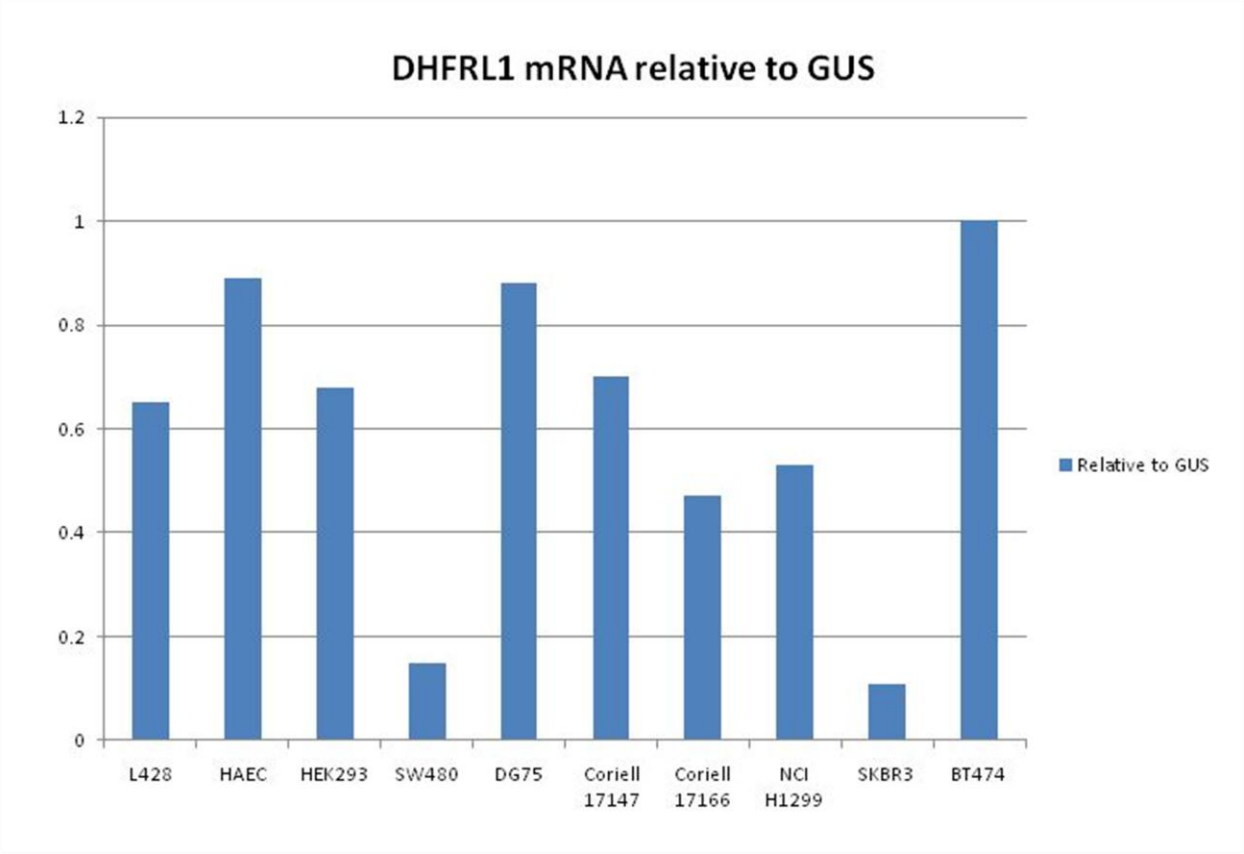


1kb ladder

Appendix 2.5 **DNA ladders** New England Biolab (NEB) DNA ladders were used to determine sizes of PCR products, plasmid preps and restriction digests. Either 100bp or a 1kb ladder was used depending on expected size of product.

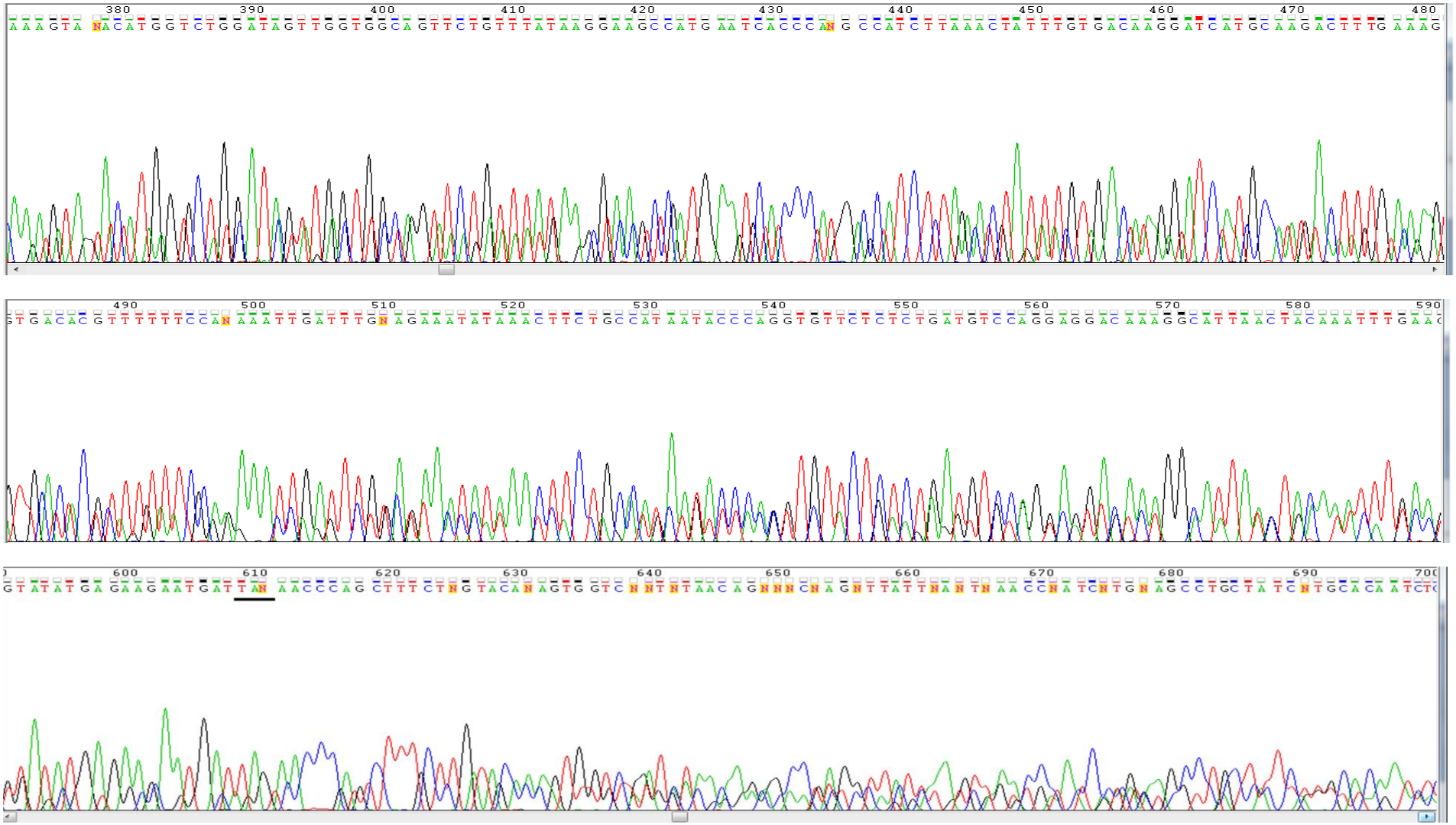


Apendix 2.6 Protein Marker. HyperPAGE (Bioline) protein marker was used to determine estimate size of protein on all SDS or Western Blot gels.

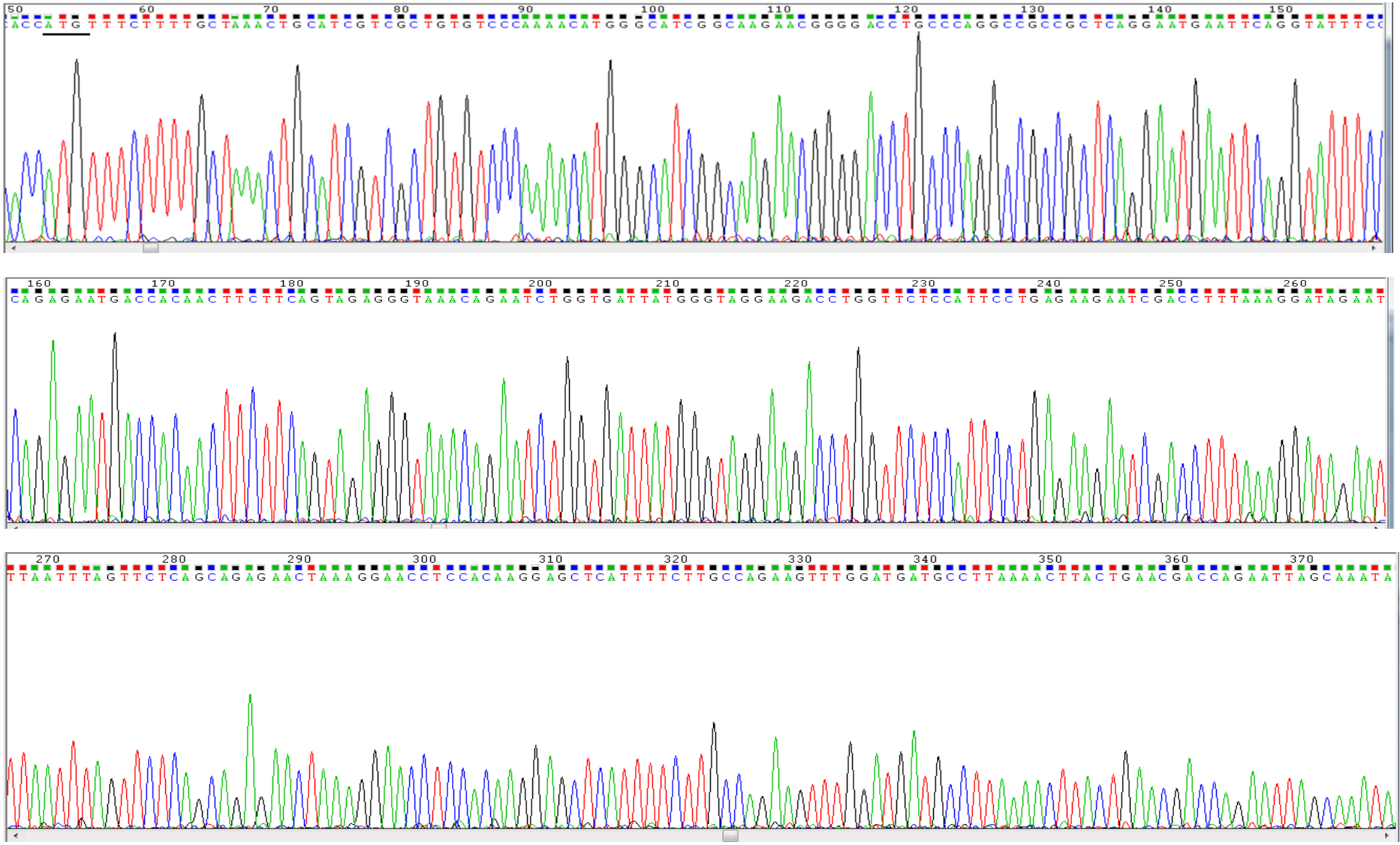


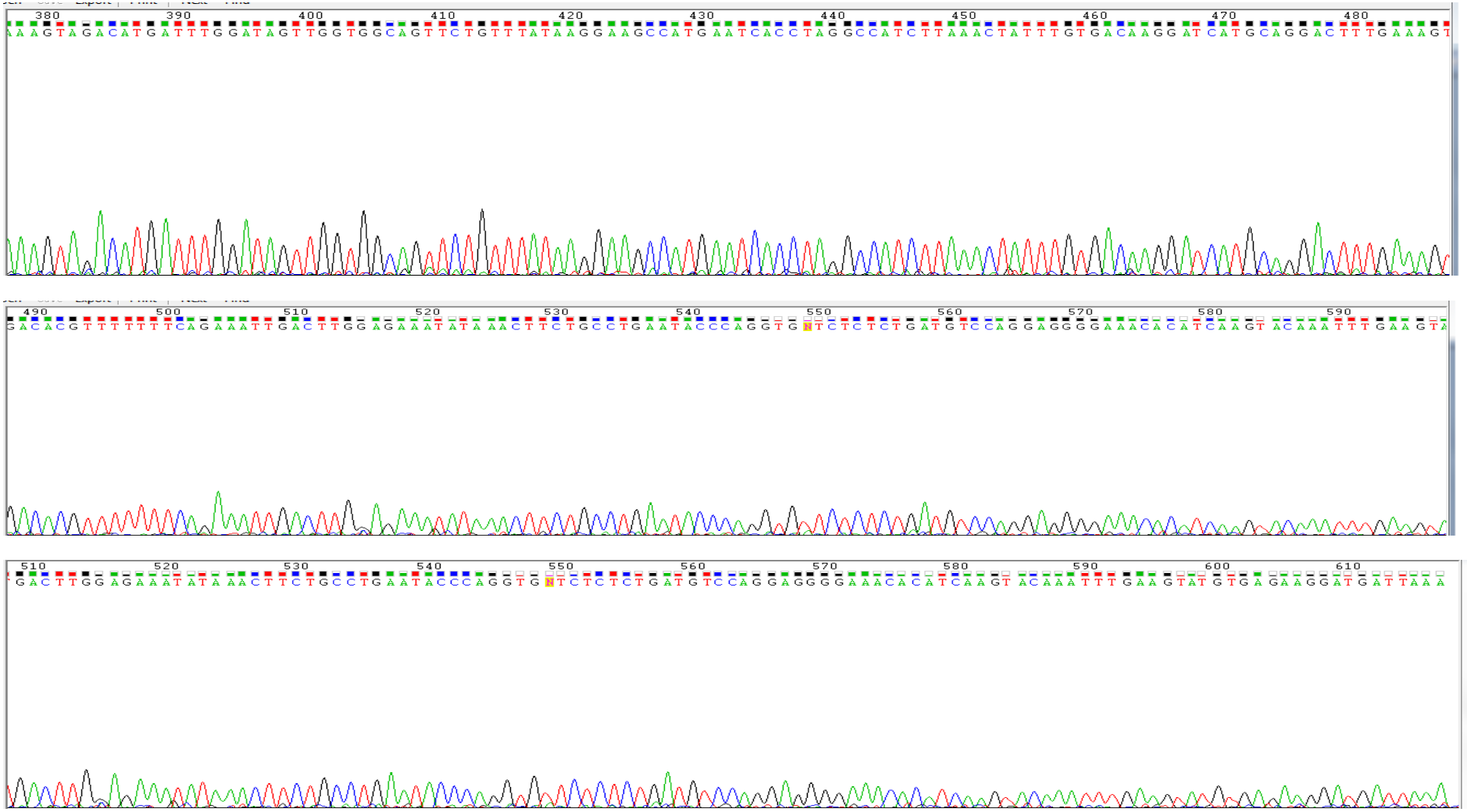
Work carried out by Dr. Kirsty O'Brien

Apendix 2.7 Confirmation of DHFRL1 expression by RT-qPCR. An RT-qPCR experiment carried out by Dr. Kirsty O Brien confirmed the expression of the T2 transcript of DHFRL1 in a number of different human cell lines, both cancerous and non-cancerous.

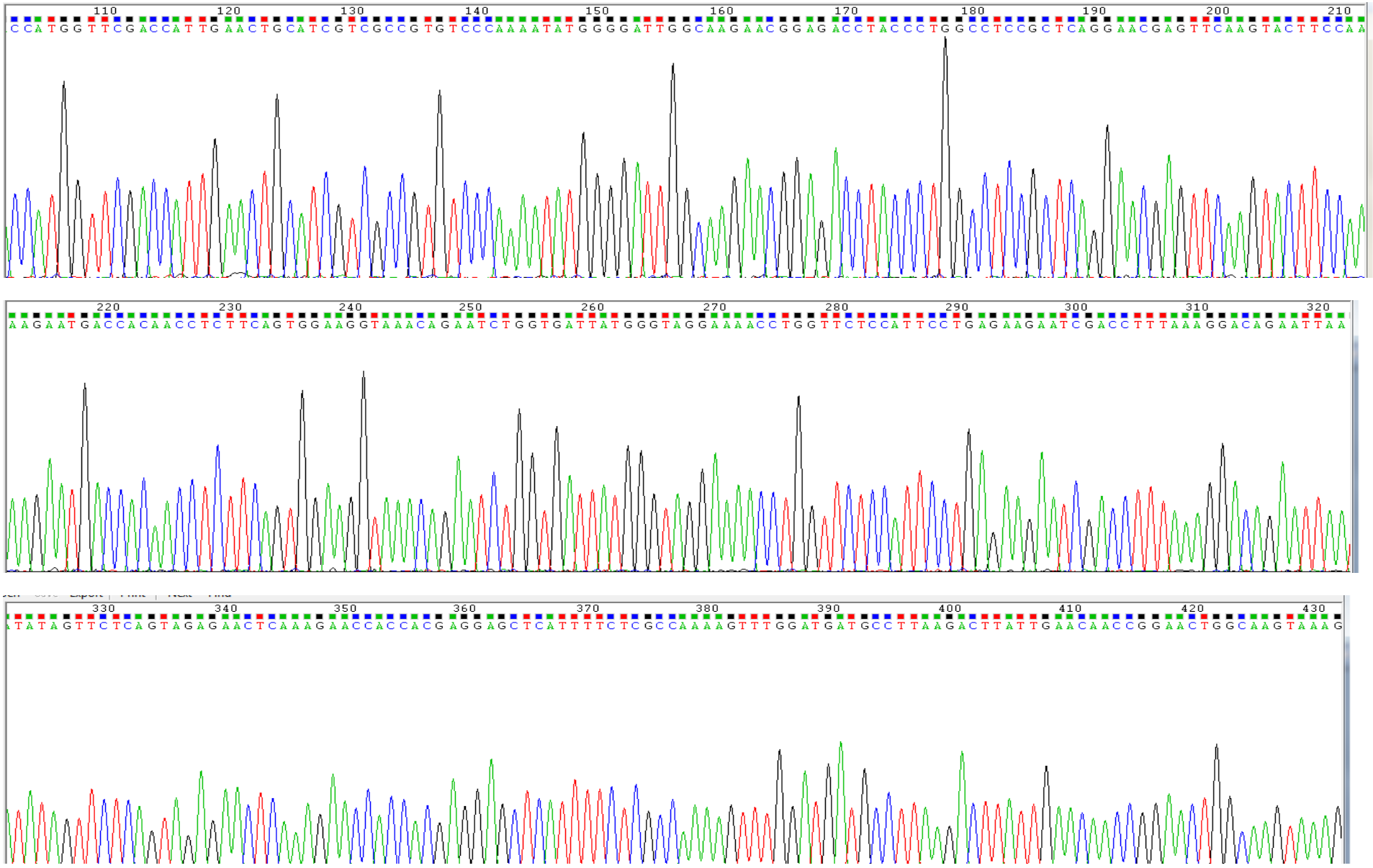


Appendix 2.8 Sanger sequencing of human DHFR entry clone





Appendix 2.9 Sanger sequencing of human DHFRL1 entry clone


110 120 130 140 150 160 170 180 190 200 210
CCATGGTTCGACCATTGAACTGCATCGTCGCCGTGTCCCAAAATATGGGGATTGGCAAGAACGGAGACCTACCCTGCCTCCGCTCAGGAACGAGTTCAAGTACTTCCAA
220 230 240 250 260 270 280 290 300 310 320
AAGAATGACCACAACCTCTTCAGTGGAAGGTAAACAGAATCTGGTGATTATGGGTAGGAAAACCTGGTTCTCCATTCCTGAGAAGAATCGACCTTTAAAGGACAGAATTAA
330 340 350 360 370 380 390 400 410 420 430
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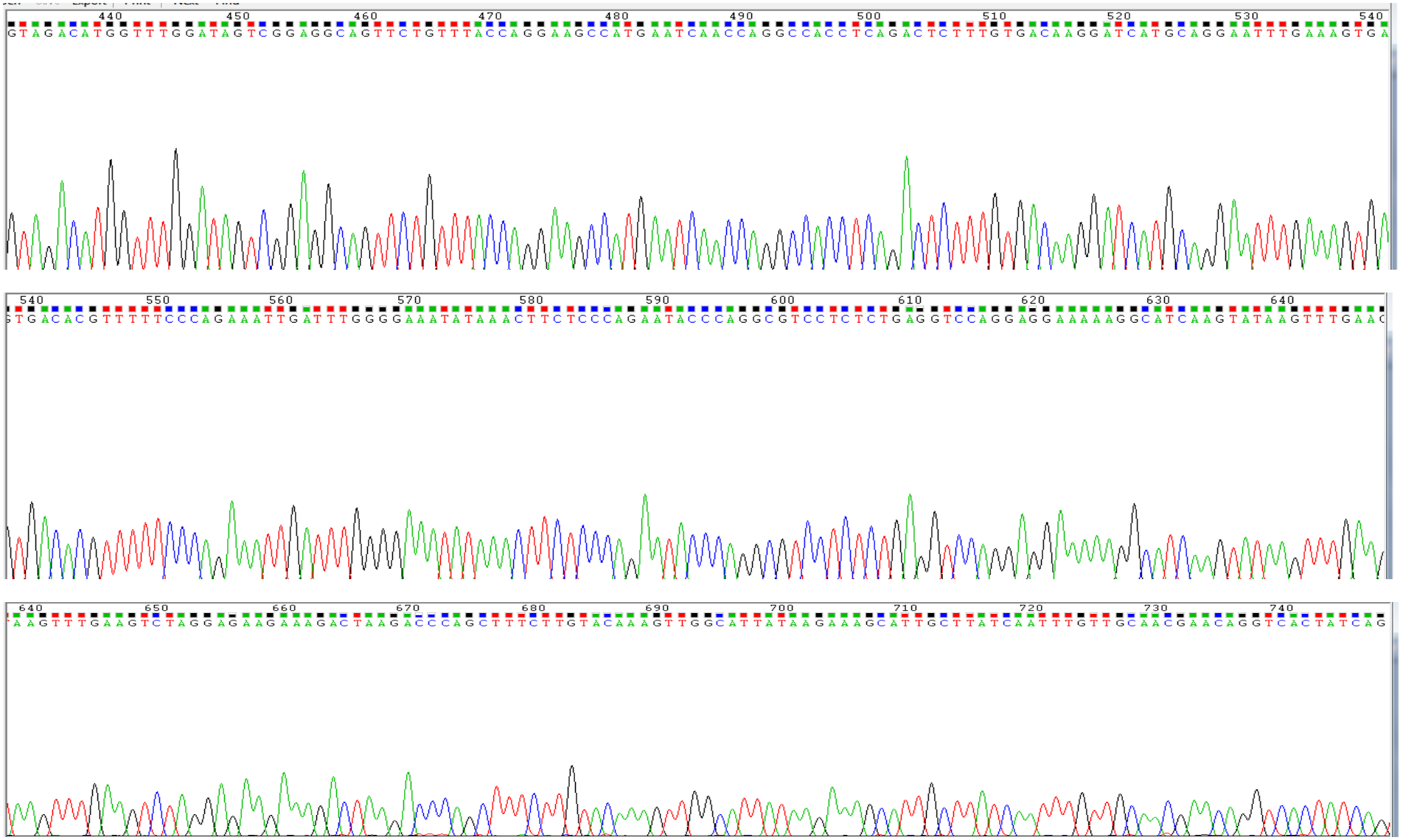
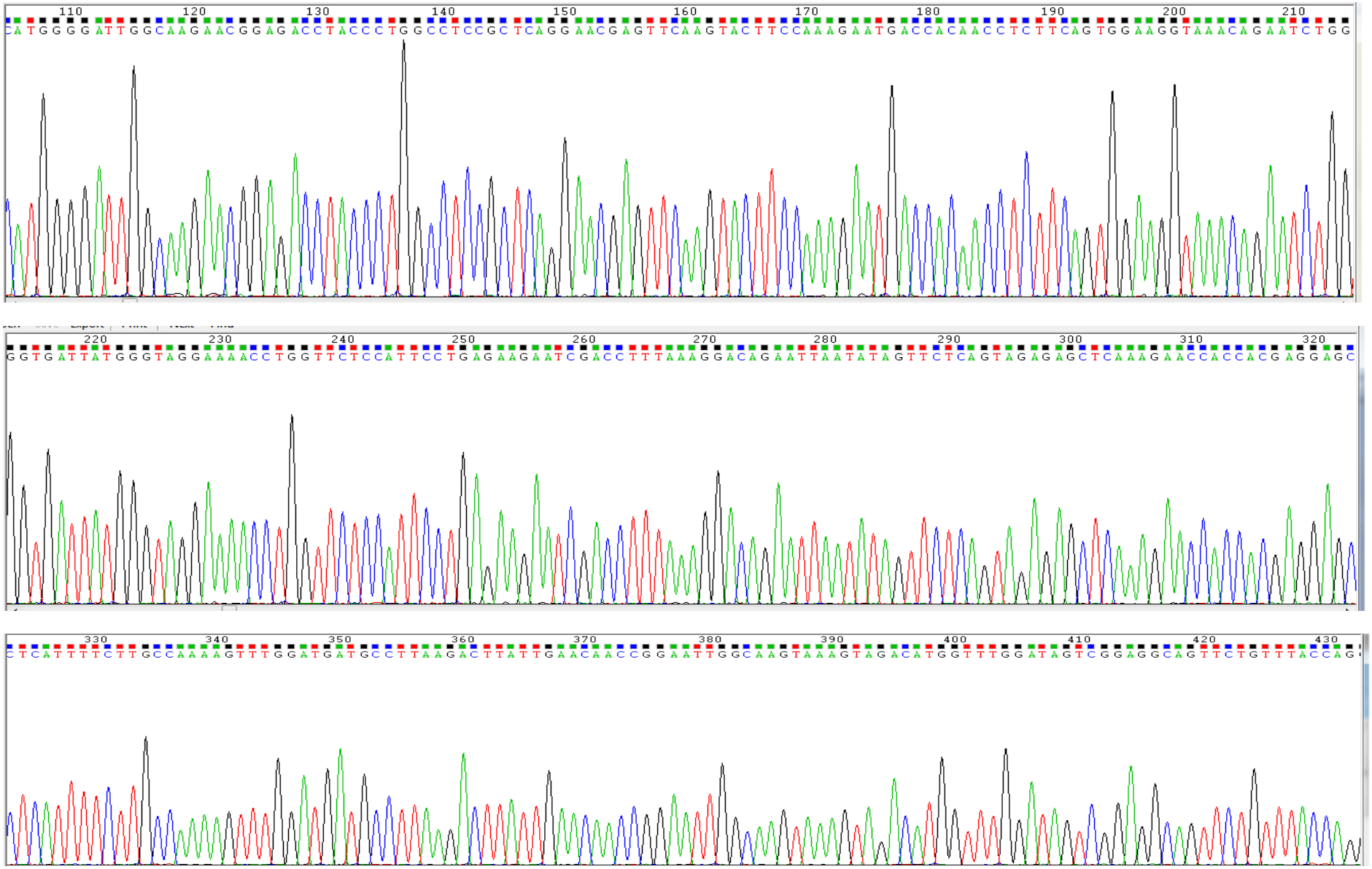


Appendix 2.10 Sanger sequencing for mouse DHFR entry clone


110 120 130 140 150 160 170 180 190 200 210
CATGGGGATTGGCAAGAACGGAGACCTACCCTGGCCTCCGCTCAGGAACGAGTTCAAGTACTTCCAAAGAATGACCACAACCTCTTCAGTGGAAGGTAAACAGAATCTGG
220 230 240 250 260 270 280 290 300 310 320
GGTGATTATGGGTAGGAAAACCTGGTTCTCCATTCCTGAGAAGAATCGACCTTTAAAGGACAGAATTAATATAGTTCTCAGTAGAGAGCTCAAAGAACCACCACGAGGAGC
330 340 350 360 370 380 390 400 410 420 430
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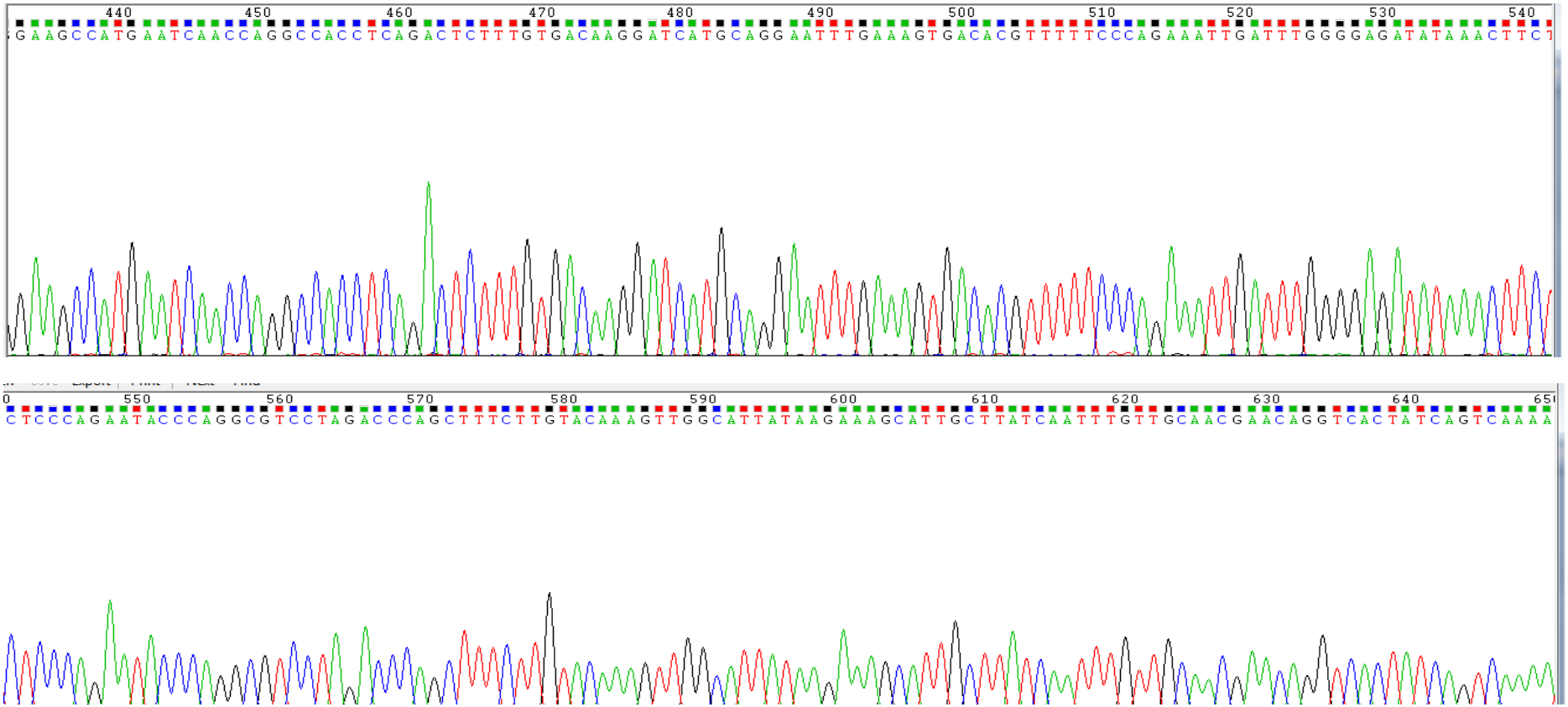
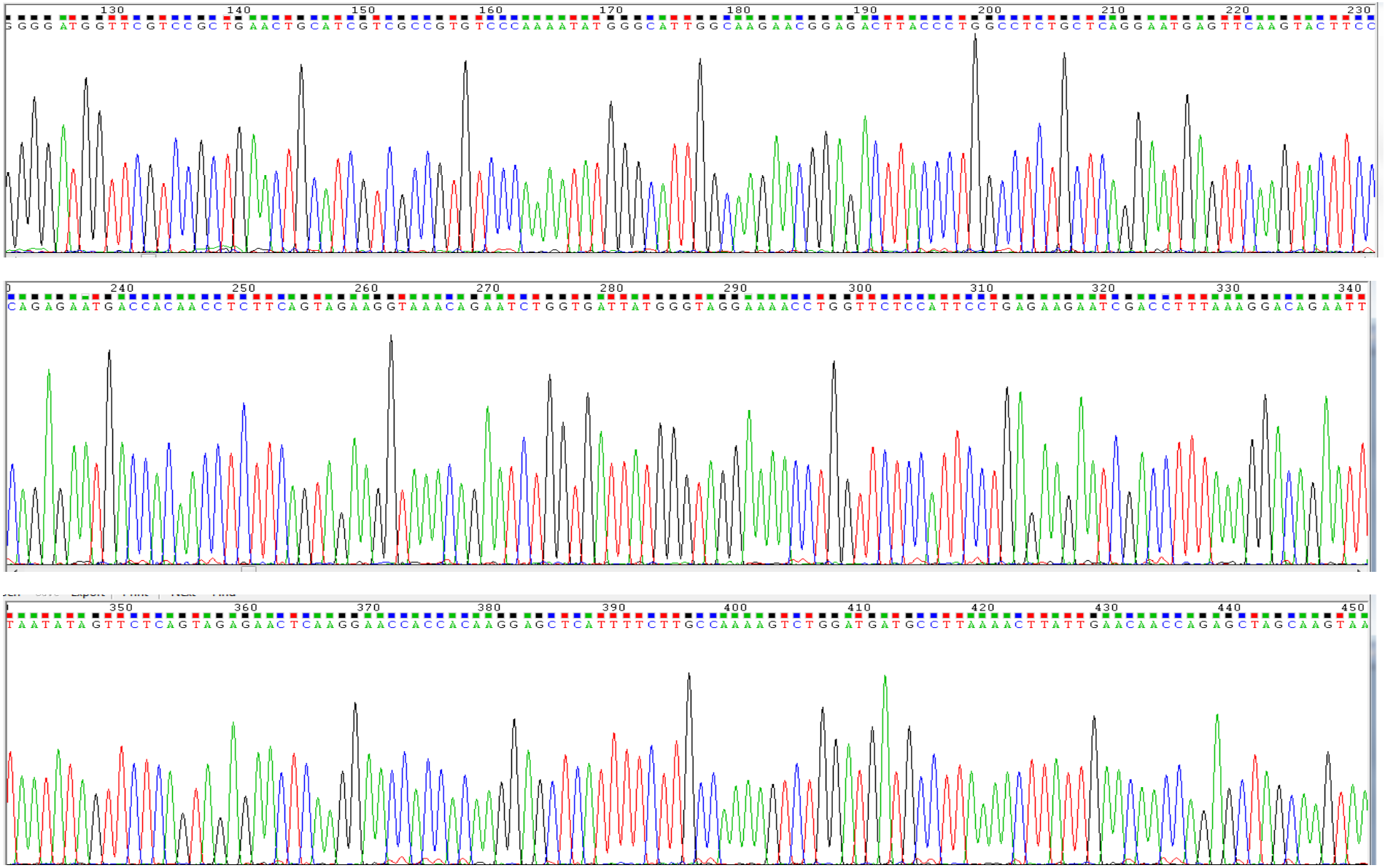


Appendix 2.11 Sanger sequencing of mouse DHFRLS entry clone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GGGGATGGTTCGTCCGCTGAACTGCATCGTCGCCGTGTCCCAAAAATATGGGCATTGGCAAGAACGGAGACTTACCCTGCCTCTGCTCAGGAATGAGTTCAAGTACTTCC
240 250 260 270 280 290 300 310 320 330 340
CAGAGAATGACCACAACCTCTTCAGTAGAAGGTAAAACAGAATCTGGTGATTATGGGTAGGAAAACCTGGTTCTCCATTCCTGAGAAGAATCGACCTTTAAAGGACAGAATT
350 360 370 380 390 400 410 420 430 440 450
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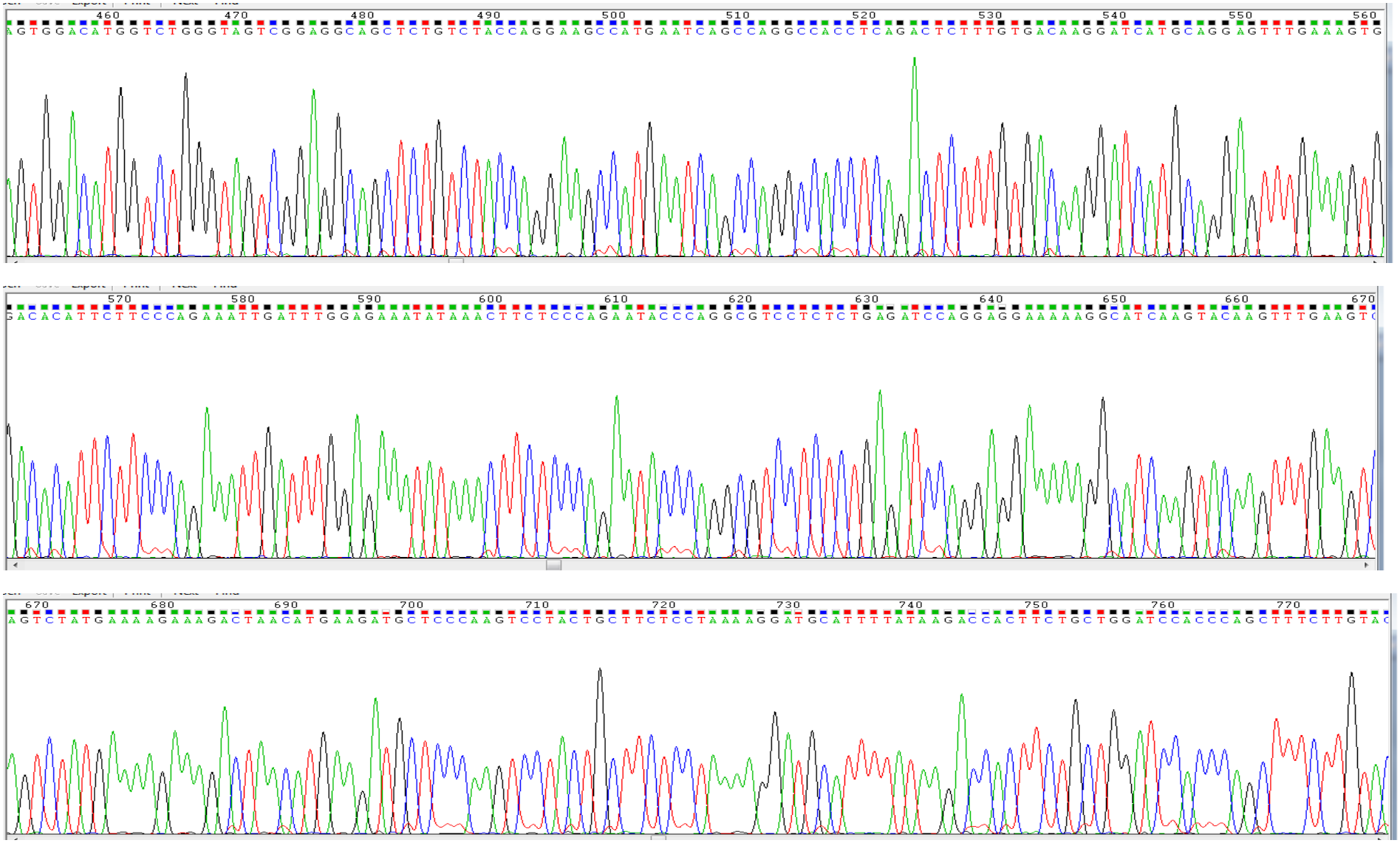
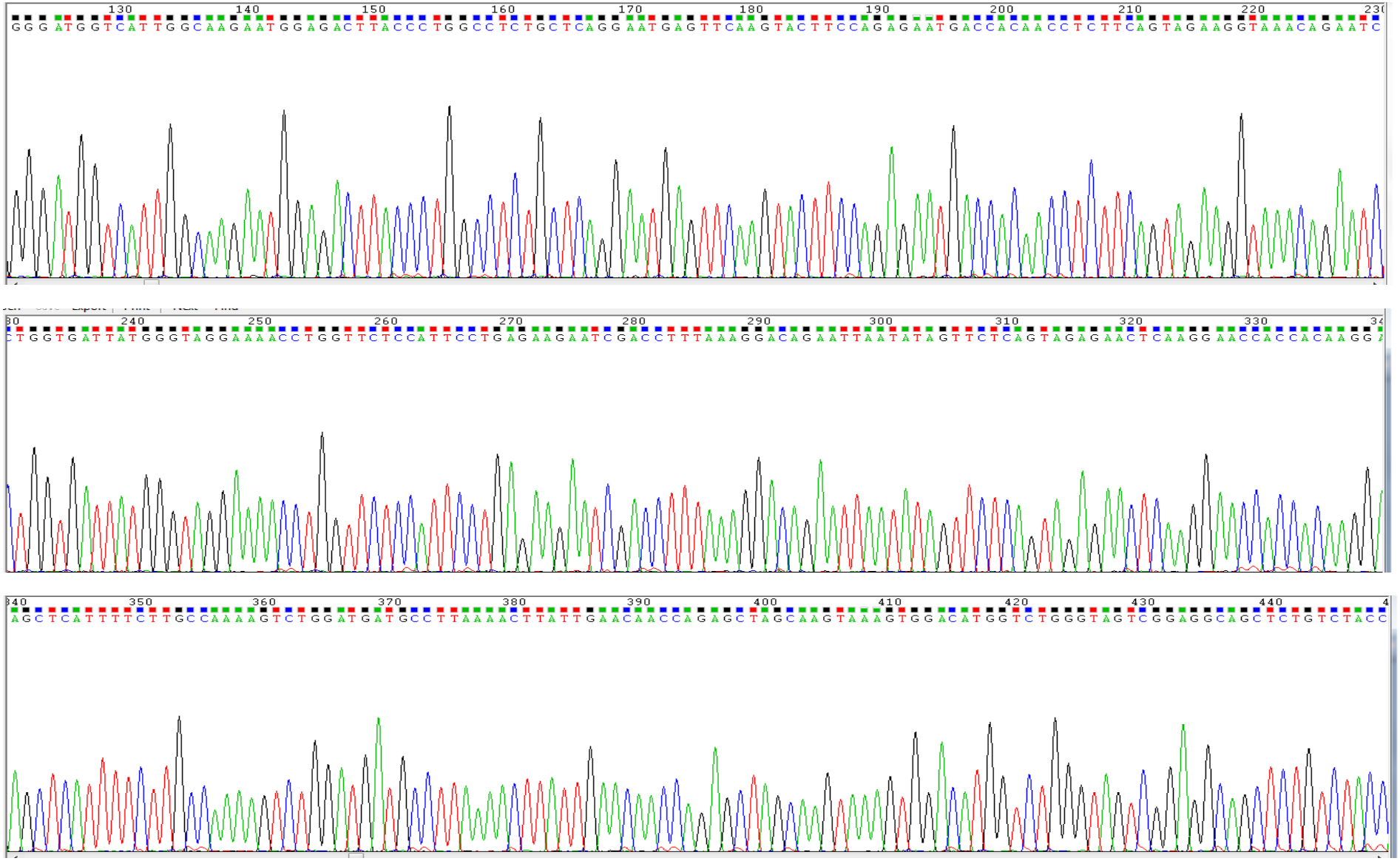


Appendix 2.12 Sanger sequencing of rat DHFR entry clone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240 250 260 270 280 290 300 310 320 330
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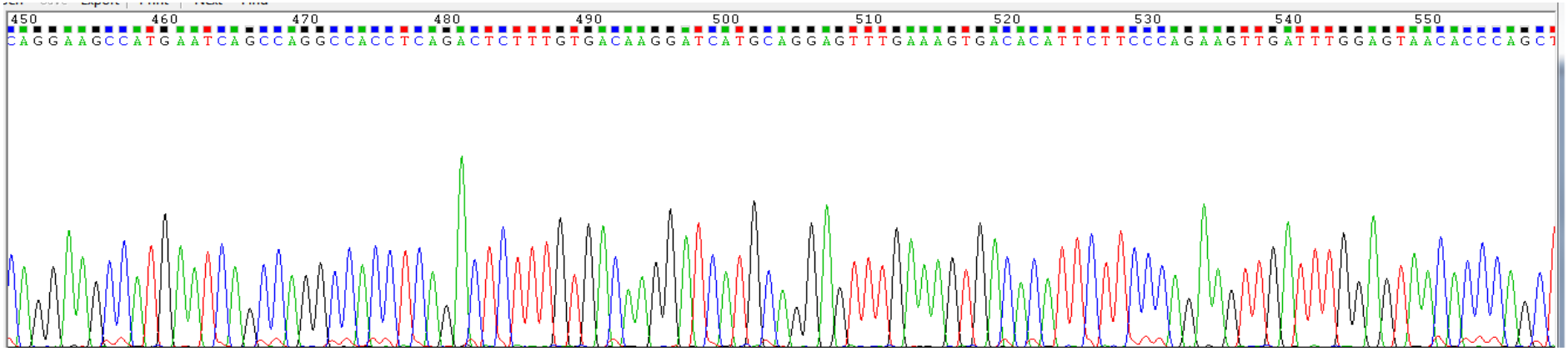

Appendix 2.13 Sanger sequencing of rat DHFRLS entry clone

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