NORTH-WEST UNIVERSITY
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The implementation of the molecular characterisation of 3-methylcrotonyl-CoA carboxylase deficiency in South Africa

BY

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Dissertation submitted in partial fulfilment of the degree Magister Scientiae in Biochemistry at the North-West University, Potchefstroom Campus

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The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF.

December 2006

Sola gratia

ACKNOWLEDGEMENTS

Firstly to my Father in heaven thank you for the courage strength and determination I receive in abundance.

Prof. P.J. Pretorius thank you for your time and effort, I appreciate every moment of your precious time and dedication to this study.

Prof. A.A van Dijk (Albie), without you this study could never be completed. Thank you for the opportunity to explore the little wonders of the human body. My appreciation towards your guidance, dedication and every minute of your time spent to this study can not be emphasised enough. Great thanks to you and NRF for the financial support.

Prof. H.J. Kotze, thank you for helping with the English language. I appreciate it.

I would like to thank Dr. Matthias Baumgartner for sharing the primer` sequences.

Ds. H.G. Schutte, thanks for your participation in this study. Without your agreement of donating a skin biopsy Chapter 5 not would be accomplished. Thank you for your contribution and participation towards the success of this study.

I want to thank my fellow students for the support and encouragement I received during this study. With you around, work is so much more worth it.

To my dearest friend Rianda, you were always there when I need a shoulder to cry on. Your inspiration means the world to me. Thank you for your friendship, encouragement and prayer, without your support everything would be so much harder to achieve.

My family thank you for all the encouragement, support and understanding I received from you. A special thanks to my grandmother for her prayer and confidence in my potential. To mom, dad and Henno, thanks for all the sacrifices you made. Thanks for the understanding encouragement and support, I love you.

ABSTRACT

The perception is that inborn errors of metabolism (IEM) are rare, but the reality is that more than 600 IEMs are now recognized. The organic aciduria, 3-methylcrotonyl-CoA carboxylase (MCC) deficiency arises when 3-methylcrotonyl-Coenzyme A (CoA) carboxylase that participates in the fourth step of the leucine catabolism is defective. Tandem mass spectrometry (MS/MS) based screening programmes in North America, Europe and Australia, showed that MCC deficiency is the most frequent organic aciduria detected, with an average frequency of 1:50 000. Therefore MCC deficiency is considered an emerging disease in these regions. The incidence of MCC deficiency in the Republic of South Africa (RSA) is not yet known. However, one 48 year old male Caucasian individual (HGS) was diagnosed suffering from mild MCC deficiency, since elevated levels of 3-hydroxyisovaleric acid, 3-hydroxyisovalerylcarnitine, 3-methylcrotonylglycine was present in his urine.

Several groups are currently working on various aspects of this emerging disease with the focus on the molecular characterisation of MCC deficiency. In the RSA no molecular based diagnostic method which complements MS/MS screening programmes have yet been implemented. Therefore, the aim of this study was to implement the necessary techniques for the molecular characterisation of MCC deficiency, the determination of the sequence of the open reading frame (ORF) of *mccA* and *mccB* subunits to determine which mutation(s) are present in the South African MCC deficient patient.

For the implementation of the molecular characterisation, a two-pronged approached was used to characterize MCC of a MCC non-deficient individual (CFC). This approach included the reverse transcriptase polymerase chain reaction (RT-PCR) amplification of the ORFs of the associated genes [*mccA* (19 exons) and *mccB* (17 exons] and the PCR amplification of selected (genomic deoxyribonucleic acid (gDNA) regions (exons *mccA*8, *mccA*11, *mccB*5, *mccB*6 and *mccB*5-intron 5-6 exon 6 (*mccB*5-6) which have been found to have mutations associated with MCC deficiency in Caucasians.

The sequence analyses produced surprising results of the amplified ORFs (CFC*mccA* and CFC*mccB*) of the MCC non-deficient individual CFC. A non-synonymous single nucleotide polymorphism (SNP) (1391C→A, H464P) associated with MCC deficiency (Gallardo *et al.*, 2001) was identified in the CFC*mccA* subunit. Another SNP (1368G→A, A456A) recently listed in GenBank was observed in the amplified CFC*mccB* ORF. No significant novel variations or described mutations were identified in the amplified genomic regions *mccA*8, *mccA*11, *mccB*5, *mccB*6 and *mccB*5-6.

The implemented molecular approach was used to characterise MCC of our MCC deficient patient (HGS). The patient did not have any mutation in the four selected exons *mccA*8, *mccA*11, *mccB*5, *mccB*6 or the genomic region *mccB*5-6. The RT-PCR amplification of both ORFs (HGS*mccA* and HGS*mccB*) resulted in multiple amplicons. Gel extracted amplicons of the expected size were sequenced. Of the 36 exons, 34 exons were sequenced. This includes all 19 exons of HGS*mccA* and 15 of 17 exons of HGS*mccB* (exons 1-6 and exons 9-17).

The non-synonymous SNP (1391C→A, H464P) detected in CFC*mccA* (MCC non-deficient individual), seems to be present in the HGS*mccA* subunit of the MCC deficient individual, HGS. The HGS*mccB* amplicons could not be entirely sequenced. However, the region exon 1-6 and 9-17 was sequenced but no described or novel mutations were identified. The lack of sequence data of region exon 7-8 led to an incomplete molecular characterisation of the MCC deficiency in HGS.

In conclusion, the basic methods and techniques for the molecular characterisation of MCC deficient patients have been implemented locally. A few additional sequencing primers need to be designed to cover *mccB*7 and *mccB*8 as well as the entire coding and non-coding strands of each MCC gene (*mccA* and *mccB*). The primers for RT-PCR of both *mccA* and *mccB* need to be further refined to ensure better specificity.

Key words: *3-Methylcrotonyl-CoA carboxylase (MCC) deficiency, mccA, mccB, molecular characterisation, inborn error of metabolism (IEM), Republic of South Africa (RSA), and Caucasian.*

OPSOMMING

Die persepsie bestaan dat aangebore metaboliese defekte skaars is, maar in werklikheid is daar meer as 600 metaboliese defekte bekend. Die organiese suururie, 3-metielkrotoniel-KoA karboksilase (MKK) defek ontstaan wanneer 3-metielkrotoniel-KoA karboksilase, wat betrokke is in die vierde stap van die leusien katabolisme, defektief is. Tandem massa-spektrometrie (MS/MS) gebaseerde neonatale siftingsprogramme in Noord-Amerika, Europa en Australië toon dat MKK die mees prevalente organiese suururie is met 'n voorkoms van 1:50 000. Daarom word MKK beskou as 'n opkomende defek in hierdie streke. Die voorkoms van MKK in Suid-Afrika is tot op hede nog nie bekend nie. Daar is wel een 48 jarige blanke man (HGS) biochemies, op grond van verhoogde vlakke van 3-hidroksieisovaleriaansuur, 3-hidroksieisovalerielkarnitien en 3-metiel-krotonielglisien in sy uriene, gediagnoseer met 'n milde vorm van MKK.

Verskeie groepe bestudeer tans verskillende aspekte van hierdie opkomende defek met die fokus op die molekulêre karakterisering van MKK. In die RSA is daar geen geïmplementeerde molekulêr gebaseerde metodes wat MS/MS siftings programme komplementeer nie. Daarom is die doel van hierdie studie om die nodige tegnieke vir die molekulêre karakterisering van MKK te implementeer. Die volgende benaderingswyse is gevolg: die bepaling van die nukleotidvolgorde van die oop leesraam (OLR, "open reading frame, ORF") van die MKK gene, nl. *mkk*A en *mkk*B, en ook om die mutasie teenwoordig in die RSA MKK pasiënt te identifiseer.

Vir die implementering van die molekulêre karakterisering van MKK is 'n tweeledige aanslag gebruik om MKK van 'n nie-defektiewe individu (CFC) te karakteriseer. Hierdie aanslag sluit in die omgekeerde transkriptase polimerase kettingreaksie (OT-PKR, "reverse transcriptase polymerase chain reaction, RT-PCR") amplifisering van die geassosieerde gene mkkA (19 eksons) en mkkB (17 eksons) se oop leesraam, asook die amplifisering van geselekteerde genomiese deoksiribonukliënsuur (gDNS, "gDNA, deoxynucliec acid") gebiede (eksons mkkA8, mkkA11, mkkB5, mkkB6 en mkkB5-6). Hierdie geengebiede is bestudeer omdat die meeste mutasie wat in blankes waargeneem is, in hierdie gebiede aangetref is.

Die volgorde analise het verrassende resultate ten opsigte van die OLR van die kontrole (CFCmkkA en CFCmkkB) individu gelewer. 'n Nie-sinonieme enkel-nukleotied polimorfisme (ENP, "single nucleotide polimorphism, SNP"; 1391C→A, H464P) wat met MKK geassosieer word, is in CFCmkkA waargeneem. 'n Ander ENP (1368G→A, A456A), wat onlangs eers in GenBank gelys is, is in CFC*mkk*B waargeneem. Geen nuwe variasie of reeds beskryfde mutasie is in die geamplifiseerde eksons waargeneem nie.

In die geval van die MKK-pasiënt (HGS) is geen mutasies in die geselekteerde eksons *mkk*A8, *mkk*A11, *mkk*B5, *mkk*B6 of die genomiese gebied *mkk*B5-6 met bogenoemde tegnieke waargeneem nie. Die OT-PKR amplifisering van beide OLRe (HGS*mkk*A en HGS*mkk*B) het gelei tot meervoudige amplikons. Amplikons van die verwagte grootte was geëkstraheer en die sekwens is bepaal. Van die 36 eksons is die sekwens van 34 eksons bepaal, dit sluit in al 19 eksons van HGS*mkk*A en 15 van die 17 eksons van HGS*mkk*B (eksons 1-6 en 9-17).

Dit wil voorkom of die nie-sinonieme ENP (1391C→A, H464P) wat in CFC*mkk*A (nie-defektiewe MKK individu) waargeneem is ook teenwoordig is in HGS*mkk*A van die defektiewe MKK individu (HGS). Die volledige nukleotied volgorde van die HGS*mkk*B kon nie bepaal word nie. Eksons 1-6 en 9-17 se nukleotied volgorde is bepaal, maar geen nuwe of gedokumenteerde mutasies is egter waargeneem nie. Die tekort aan volgorde data van gebied ekson *mkk*B7 en *mkk*B8 het gelei tot onvolledige molekulêre karakterisering van MKK in HGS.

Om saam te vat, die basiese molekulêre metodes en tegnieke vir die karakterisering van MKK in MKK-defektiewe pasiënte is plaaslik suksesvol geïmplementeer. Nuwe voorvoerders moet ontwerp word om *mkk*B7 en *mkk*B8 sowel as die volledig-koderende en nie-koderende stringe van elke MKK-geen (*mkk*A en *mkk*B) se volgorde te kan bepaal Die voorvoerders vir OT-PKR van beide *mkk*A en *mkk*B moet ook verder verfyn word om beter spesifisiteit te verseker.

Sleutelwoorde: *3-Metielkrotoniel-KoA karboksilase (MKK) defek, mkkA, mkkB, molekulêre karakterisering, aangebore metaboliese defek, Republiek van Suid-Afrika (RSA) en Blank.*

TABLE OF CONTENTS

ABBREVIATIONS & SYMBOLS

A

α	Alpha
A, Ala	Alanine
A, a	Adenine, a purine nucleotide
AMV	Avian Myeblastosis virus
ATP	Adenosine triphosphate
A_{260}/A_{280}	Ratio of absorbance measured at 260 nm and 280 nm
ac	acceptor
ACC	Acetyl-CoA carboxylase
ADP	Adenosine diphosphate
AD	Autosomale dominant
AGPC	Acid guanidinium thiocyanate-phenol-chloroform
AR	Autosomal recessive

B

β	Beta
B, Asx	Asparagine, aspartic acid
bp	Base pairs (nucleotides)

C

C, c	Cytosine, a pyrimidine nucleotide
C, Cys	Cysteine
°C	Degree Celsius
cDNA	Complementary Deoxyribonucleic acid
cm^2	Square centimetre
CoA	Coenzyme A
CO_2	Carbon dioxide

D

D, Asp	Aspartic acid
d	Day
ddH_2O	Double distilled water
dT	Deoxy thymine
dNTP	Deoxynucleotide triphosphate
ddNTP	2',3'-dideoxynucleoside triphosphate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsDNA	double stranded DNA
DTT	Dithiotreitol

E

E, Glu	Glutamic acid
EDTA	Ethylenediaminetetraacetic acid
e.g.	*exempli gratia*, for example

ABBREVIATIONS & SYMBOLS
CONTINUE

etc.	*et cetera*, and so forth
et al.	*et altera*, and others
ε	Epsilon
EtOH	Ethanol
ENP	enkel nukleotied polimorfisme
F	
F, Phe	Phenylalanine
FAD	Flavin adenine dinucleotide (Oxidised form)
$FADH_2$	Flavin adenine dinucleotide (Reduced form)
For, fwd	Forward
FBS	Fetal bovine serum
FA	Formaldehyde
G	
G, Gly	Glycine
G, g	Guanine, a purine nucleotide
g	Gram (s)
g	Gravitational field (g = 9,81 $m.s^{-2}$)
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
gDNA	Genomic deoxyribonucleic acid
gDNS	Genomiese deoksiribonukluiën suur
GA-I	Glutaric acidemia type 1
H	
H, His	Histidine
H^+	Hydrogen ion
HCO_3^-	Bicarbonate ion
H_2O	Water
HMG	3-Hydroxy-3-methylglutaryl
HCS	Holocarboxylase synthetase
I	
I, Ile	Isoleucine
i.e.	*id est,* that is
IEM	Inborn errors of metabolism
K	
K, Lys	Lysine
Kb	Kilobase (1000 bp)
Kg	Kilogram
K^+	Potassium ion
L	
L, Leu	Leucine

ABBREVIATIONS & SYMBOLS

CONTINUE

Ltd.	Limited
M	
M, Met	Methionine
M	Molar
MCC	3-methylcrotony-CoA carboxylase
mM	Millimolar (1 $x10^{-3}$)
mg	Milligram (s)
mg / day	Milligram (s) per day
ml	Millilitre (s)
Mg^{2+}	Magnesium ion
$MgCl_2$	Magnesium chloride
min	Minutes
mRNA	Messenger Ribonucleic acid
MCD	Multiple carboxylase deficiency
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MKK	3-methielkrotoniel-KoA karboksilase
mccA	3-methylcrotony-CoA carboxylase alpha subunit -gene
MCCA	3-methylcrotony-CoA carboxylase alpha subunit -protein
mccB	3-methylcrotony-CoA carboxylase beta subunit -gene
MCCB	3-methylcrotony-CoA carboxylase beta subunit -protein
*mkk*A	3-methielkrotoniel-KoA karboksilase alfa subeenheid -geen
*mkk*B	3-methielkrotoniel-KoA karboksilase beta subeenheid -geen
MKKA	3-methielkrotoniel-KoA karboksilase alfa subeeneheid- proteïen
MKKB	3-methielkrotoniel-KoA karboksilase beta subeeneheid- proteïen
MCADD	Medium chain acyl-CoA dehydrogenase Deficiency
MOPS	3-[N-morpholino] propanesulfonic acid
N	
N, Asn	Asparagine
NaCl	Sodium chloride
NAD^+	Nicotinamide adenine dinucleotide (oxidised)
NADH	Nicotinamide adenine dinucleotide (reduced)
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidised)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
No.	Number

ABBREVIATIONS & SYMBOLS

CONTINUE

NWU	North-West Univeristy
nm	nanometre
#	Number
O	
O_2	Oxygen
OH^-	Hydroxy ion
ORF	Open reading frame
OD	Optical density
Ω	Ohm
OT	Omgekeerde transkriptase
P	
P, Pro	Proline
PBS	Phosphate buffered saline
PCC	Propionyl-CoA carboxylase
PCR	Polymerase chain reaction
pH	Percentage hydrogen
pmol	Picomol
P	Phosphate
PKR	Polimerase ketting reaksie
PC	Pyruvate carboxylase
PKU	Phenyl ketonuria
%	Percent
Q	
Q, Gln	Glutamine
R	
R, Arg	Arginine
RNA	Ribonucleic acid
RT	Reverse transcriptase
®	Registered
rRNA	Ribosomal ribonucleic acid
rev	Reverse
S	
S, Ser	Serine
S.A.	South Africa
Sec.	Seconds
SNP	Single nucleotide polymorphism
SDS	Sodium dodecyl sulphate
SCADD	Short chain acyl-CoA dehydrogenase deficiency

ABBREVIATIONS & SYMBOLS

CONTINUE

T	
T, Thr	Threonine
TAE	Tris acetic acid ethylenediaminetetraacetic acid
Tris®	Tris (hydroxymethyl) aminomethane
T, t	Thymine, a pyrimidine nucleotide
Tm	Calculated melting temperature
™	Trademark
tRNA	Transport ribonucleic acid
U	
µ	Micro
µM	Micro molar
µl	Microliter
µg	Microgram (s)
UV	Ultra violet
USA	United States of America
UTR	Untranslated region
V	
V, Val	Valine
V	Volt
VLCADD	Very long chain acyl-CoA dehydrogenase deficiency
W	
W, Trp	Tryptophan
WBC	White blood cells
X	
XD	X-linked dominant
XR	X-linked recessive
Y	
Y, Tyr	Tyrosine
Z	
Z, Glx	Glutamine or glutamic acid
OTHER	
18S rRNA	Ribosomal ribonucleic acid from the 40S
28S rRNA	Ribosomal ribonucleic acid from the 60S

LIST OF EQUATIONS

LIST OF FIGURES

LIST OF FIGURES

CONTINUE

LIST OF FIGURES
CONTINUE

LIST OF TABLES

LIST OF TABLES
CONTINUE

Appendixes

Chapter One

Introduction

Inborn errors of metabolism (IEM) were thought to be rare (Chen and Hofestad 2005; Marsden *et al.,* 2006), but more than 600 human diseases due to IEM are now recognised (Anon, 2006). This number is steadily increasing, as techniques become more sensitive for identifying relevant biochemical metabolites. One of the diseases considered an emerging disease is 3-methylcrotonyl-CoA carboxylase (MCC) deficiency. Screening programmes in North America (Gibson *et al.,* 1998; Naylor and Chace, 1999; Smith *et al.,* 2000), Europe (Roscher *et al.,* 2000) and Australia (Wilcken *et al.,* 2000), showed that MCC deficiency is the most frequent organic aciduria detected in tandem mass spectrometry (MS/MS) based newborn screening (NBS) programmes. The average frequency is 1:50 000 (Baumgartner *et al.,* 2001). Although MCC deficiency is regarded as the most frequent organic aciduria in North America, Europe and Australia, the incidence of MCC deficiency in South African is unknown. The metabolic unit of the School of Biochemistry of the North-West University identified one 48 year adult male (HGS) with MCC deficiency. Analyses of the urine metabolite profile confirmed a late onset MCC deficiency.

Several groups are currently working on various aspects of this emerging disease. The focus is on the molecular basis of MCC deficiency specifically on the biotin responsiveness, the correlation of genotype-phenotype characteristics and the correlation of the residual enzyme activity to the clinical outcome of the patient (Baumgartner *et al.,* 2001, Gallardo *et al.,* 2001, Holzinger *et al.,* 2001, Dantas *et al.,* 2005). Mitochondrial signal peptide studies were also done (Stadler *et al.,* 2005), but no correlations have been found.

For South Africa (RSA) to catch up and elevate itself to the forefront in biochemical and molecular screening it is necessary to study recent literature in order to understand the field and to implement similar studies in the RSA (Chapter 2). The aim of this study was to implement and used the appropriate molecular techniques to characterize the molecular profile of MCC deficiency in RSA. Implementation of the characterisation involves the optimisation of techniques using cultured fibroblast cells of a MCC non-deficient individual. The implementation of mature mRNA transcripts was done (chapter 3) as well as on the genomic level (chapter 4). These techniques were then applied to characterize the two MCC genes (*mccA* and

mccB) of the MCC deficient patient on cDNA as well as on the genomic level (Chapter 5). The results of this study are presented in the various chapters (Chapter 3, 4 and 5), with the final conclusions and suggestions for future studies are discussed in Chapter 6.

Chapter Two

Literature review

2.1 INTRODUCTION

Metabolism can be defined as the sum total of all the chemical reactions in a living cell and is generally divided into anabolism and catabolism. Anabolism refers to the synthesis of complex molecules from simpler molecules with the input of energy. Catabolism on the other hand is the breakdown of larger, more complex molecules into simpler molecules with the release of energy (Prescott *et al.*, 2002). Since metabolism plays a major role in maintaining homeostasis in the human body any factor that interferes with the progress and control of any metabolic process and thus homeostasis will predispose into a metabolic disorder.

People are currently more aware of a healthy lifestyle and a good quality of life. Sportsmen and sportswomen want to reach their full potential. Parents want their children to have the best life they can provide and are concerned as to how they can contribute to limit disease such as the presentation of inherited metabolic diseases. The importance of the metabolism is thus enormous. Knowledge of metabolism cannot be overemphasised since a metabolic disease is determined by the metabolic profile of the individual (Gallagher, 2005).

Gregor Johann Mendel (1822 - 1884) and Sir Archibald Garrod (1857 - 1936) made enormous contributions to the development of biology. Mendel was well ahead of his time when he discovered inheritance patterns in peas (Bateson, 1902). This discovery was the basis of great breakthroughs in biology. With Mendelian inheritance patterns in mind, Sir Archibald Garrod discovered that genes that are inherited from one generation to another could affect biochemical processes and called such inherited defects "inborn errors of metabolism (IEM)" (Garrod, 1902; Garrod, 1923). Today Garrod is known as the father of inborn errors of metabolism. Like Mendel, Garrod was well ahead of his time. Therefore, his work was largely unnoticed until much later when the efforts of Beadle and Tatum established the 'one gene – one enzyme' hypothesis, which means, one gene control the production of one enzyme (Evers, 1999).

An IEM is a genetic disorder in which a specific enzyme in a metabolic pathway is affected. In IEMs, the defective gene leads to the absence of essential proteins or the synthesis of a dysfunctional protein which results in a block in a specific metabolic pathway (Figure 2.1). Due to the presence of a defective enzyme, metabolites accumulate upstream of the block in the affected metabolic pathway. This leads to the activation of alternative pathways as illustrated

in Figure 2.2 (Chen and Hofestad 2005; Lanpher *et al.,* 2006).

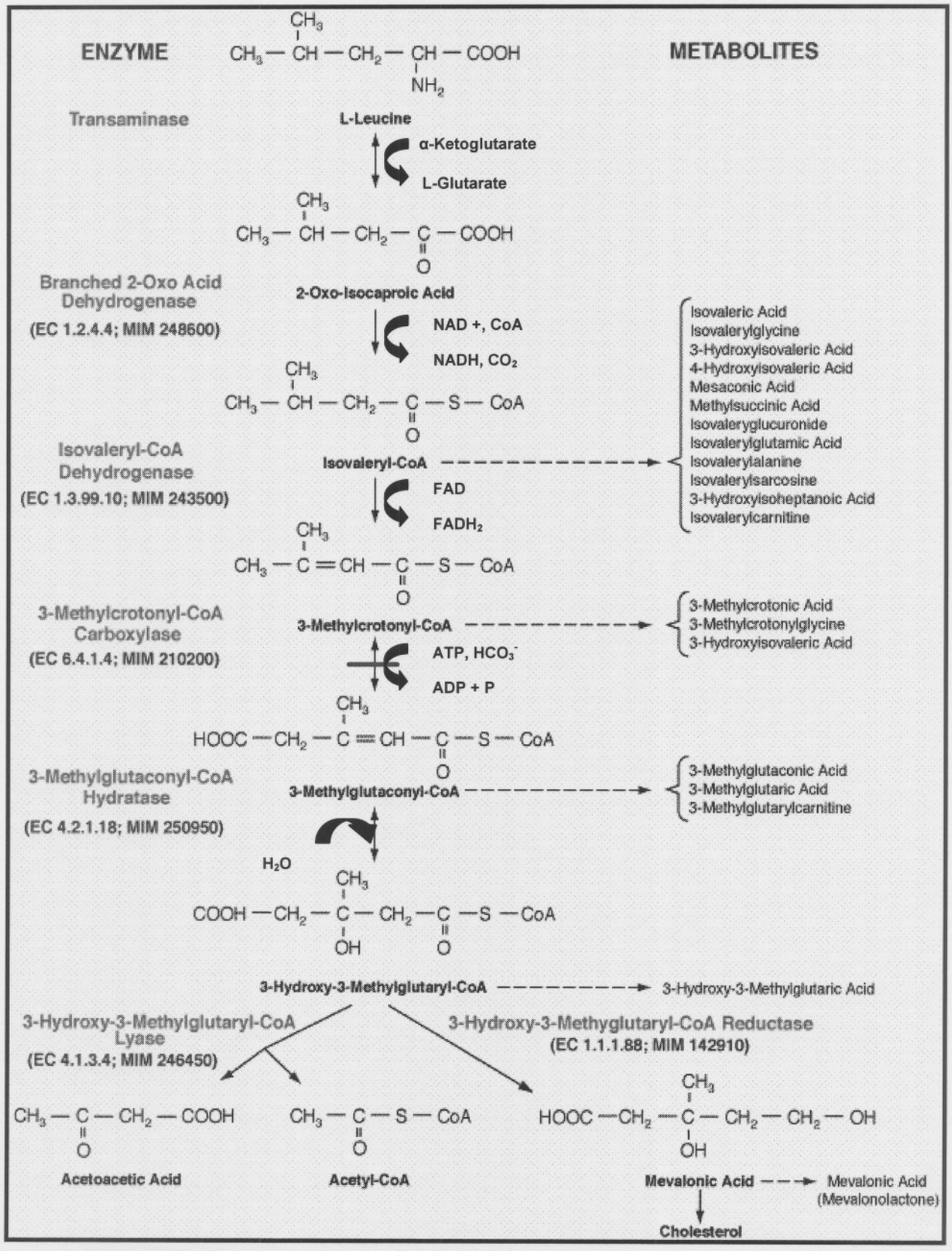


FIGURE 2.1: DEGRADATION PATHWAY OF LEUCINE ATP, Adenosine triphosphate; HCO_3^-, Bicarbonate; NAD^+, Nicotinamide adenine dinucleotide (oxidised); NADH, Nicotinamide adenine dinucleotide (reduced); CO_2, carbon dioxide; FAD, Flavin adenine dinucleotide (Oxidised form)$FADH_2$, Flavin adenine dinucleotide (Reduced form); ADP, Adenosine diphosphate; P, phosphate; CoA, coenzyme A (Modified from Sweetman and Williams, 2001 and Romero *et al.,* 2003).

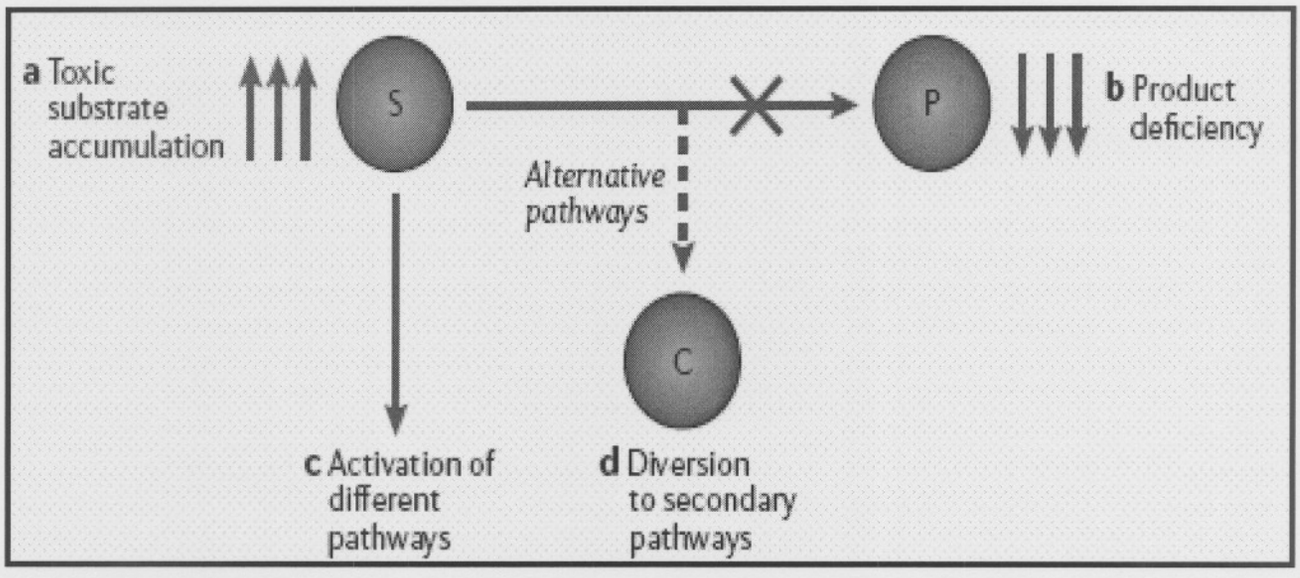


FIGURE 2.2: THE EFFECT OF AN ENZYMATIC BLOCKAGE a) direct toxicity of the accumulating upstream substrate (S); b) deficiency of the downstream product (P); c) activation of alternative pathways; diversion of metabolic flux to secondary pathways and alternative metabolite (C) production (Lanpher *et al.,* 2006).

The accumulating metabolites are generally suspected to be the cause of clinical symptoms and pathophysiology of an inherited disease. Clinical symptoms of IEMs can vary from asymptomatic to extremely severe (Scriver, 2006). Metabolic biochemical analyses are currently more frequently used to confirm diagnoses of IEMs. Furthermore, it is also important to understand the genetic cause of these diseases and to establish genetic counselling programmes for patients and their families. Molecular characterisation complements the biochemical diagnosis of IEMs (Sanne, 2005).

IEMs were thought to be rare, but as the technology and detection methods developed and improved, more and more individuals are detected and identified. Newborn screening (NBS) programmes that use MS/MS is the most widely used method for the detection of inborn errors of metabolism (Chen and Hofestad 2005; Marsden *et al.,* 2006). Despite the relative abundance of new case reports there is considerable evidence that many of these disorders still remain undetected and/or misdiagnosed (Saudubray and Charpentier, 2001).

One of the primary challenges in the classification of IEMs is their extreme phenotypic diversity. Various approaches to classify IEMs have been followed, but no single, universal classification system exists (Lanpher *et al.,* 2006). Some systems classify IEMs according to the affected organ, the affected organelle or the age at presentation. Another system classifies IEMs according to specific metabolic pathways or transport systems involved as well as the mode of inheritance. Table 2.1 gives an example of a classification system described by Mueller and Young (1995)

where the IEM of interest is categorised according to the metabolic pathway involved. The metabolic pathways are named according to the main molecule that is involved in that particular metabolism.

Disorders in the metabolism of organic acid are one of these biochemical classification categories. The IEM of interest in this study, MCC deficiency, is one of the organic acid disorders. Organic acidurias as well as the degradation of leucine will be discussed in Section 2.2.

TABEL 2.1: BIOCHEMICAL CLASSIFICATION OF THE INBORN ERRORS OF METABOLISM

Metabolic pathway affected	Mode of Inheritance	Example of disorder
Amino acid metabolism	AR	Phenylketonuria
Amino acid transport	AR	*Cystinuria*
Carbohydrate metabolism	AR	Galactosemia
Copper metabolism	AR/XR	Menkes' disease
Glycogen storage	AR	Pomp's disease
Lipoprotein metabolism	AD	Familial hypercholesterolamie
Organic acid disorders	AR	3-Methylcrotonyl-CoA carboxylase deficiency
Porphyrin metabolism	AD	Porphyria variagata
Purine / Pyrimidine metabolism	XR	Lesch-Nyhan disease
Steroid metabolism	AR	Testicular feminisation
Thyroid hormone biosynthesis	AR	Congenital hypothyroidism
Urea cycle disorder	XD	Ornithine transcarbamylase deficiency
Vitamin metabolism	XD / AR	Rickets

AD = autosomal dominant, AR = autosomal recessive; XD –X-linked dominant; XR = X linked recessive (Adapted from Mueller and Young, 1995).

2.2 ORGANIC ACIDURIA: BRANCH CHAIN AMINO ACID DEGRADATION

The degradation pathway of the branched chain amino acid leucine is of special interest since the focus of this study is on MCC deficiency. This involves a metabolic defect in the metabolism of the branched chain amino acid leucine. The other branched chain amino acids are isoleucine and valine. The degradation of these three branched chain amino acids begins with three reactions that use general enzymes, i.e. a branched chain amino acid aminotransaminase to the corresponding oxidative decarboxylation with branched-chain α-keto acid dehydrogenase to the corresponding acyl-CoA. Isovaleryl-CoA is derived from leucine, 2-methylbuteryl-CoA is derived form isoleucine and isobutyryl-CoA is derived from valine. These acyl-CoA products are then metabolised by separate pathways to intermediates which enter the general metabolism. Figure 2.1 illustrates the

degradation pathway of L-leucine (Sweetman and Williams, 2001; Romero *et al.,* 2005). Leucine degradation continues with biotin dependent MCC, 3-methylglutaconyl-CoA hydratase and 3-hydroxy-3-methylglutaconyl (HMG)-CoA lysate to yield acetyl-CoA and acetoacetate (Voet and Voet, 2004).

2.3 CLINICAL AND BIOCHEMICAL CHARACTERISTICS OF 3-METHYLCROTONYL-CoA-CARBOXYLASE DEFICIENCY

MCC is an autosomal recessive disorder of leucine catabolism, caused by a defective 3-methylcrotonyl-CoA carboxylase (EC 6.4.1.4) enzyme (Sweetman and Williams, 2001) which catalyses the fourth step in the catabolism of leucine (Figure 2.1). The first case of MCC deficiency was documented by Eldjarn (1970). MCC deficiency is also known as: 'Isolated biotin-resistant 3-methyl-crotonyl-CoA carboxylase; Isolated biotin-resistant MCC; Isolated 3-methylcrotonyl-CoA carboxylase; Isolated MCC; Methylcrotonylglycinuria' [omim 210200 and 210210].

Before the advent of MS/MS technology and the introduction of NBS programmes in the late 1990s, it was thought that MCC deficiency was an extremely rare metabolic disorder. However, the introduction of NBS programmes in North America (Gibson *et al.,* 1998; Naylor and Chace, 1999; Smith *et al.,* 2000), Europe (Roscher *et al.,* 2000) and Australia (Wilcken *et al.,* 2000) showed that MCC deficiency is the most frequent organic aciduria detected. The overall frequency of MCC deficiency is approximately 1 in 50,000 (Baumgartner *et al.,* 2001).

Clinical manifestations of MCC deficiency vary from asymptomatic to neonatal onset with severe neurological involvement and death (Finnie *et al.,* 1976; Bannwart *et al.,* 1992; Lehnert *et al.,* 1996; Gibson *et al.,* 1998; Holzinger *et al.,* 2001). Clinical presentation includes feeding difficulties, vomiting, seizures, hyper or hypotonia dermatological changes and progressive loss of consciousness, which may lead to coma and death (Gibson *et al.,* 1994). Patients who develop neurological manifestations in the first two years of life also present with severe carnitine deficiency, hypoglycemia, ketoacidosis and hyperammonemia (Holzinger *et al.,* 2001; Sweetman and Williams, 2001). Clinical decompensation can occur rapidly in an infant but may be more gradual in older individuals (Stagni, 2005). In general, the earlier the patient presents the poorer is the prognosis (Leaner *et al.,* 1996).

The metabolic profile of MCC deficient patients include elevated levels of the following conjugates: 3-hydroxyisovaleric acid, 3-hydroxyisovaleryl carnitine, 3-methylglycine and some 3-methylcrotonic acid that are excreted in urine. However, the diagnosis for MCC is complicated by the similarities in symptoms and indicator metabolites with multiple carboxylase deficiency (MCD). Figure 2.3

illustrates the biotin cycle, the four biotin dependent carboxylases and the metabolites associated with MCD. The MCD associated metabolite profile includes 3-hydroxyisovaleric acid and 3-methylglycine as well as isovalerylglycine, 3-methylglutaconic acid, 3-hydroxy-3-methylglutaric acid, lactic acids and elevated levels of methylcitric and 3-hydroxypropionic acid in the urine (Holzinger *et al.,* 2001).

Enzyme activity analyses are needed to distinguish between MCC deficiency and MCD. The activity of both MCC and holocarboxylase synthetase (HCS) should be measured to confirm the defective enzyme. MCD can result from either biotinidase deficiency or from a defective HCS enzyme. Biotinidase deficiency is usually responsive to the administration of biotin (Tsia *et al.,* 1989). The covalent binding of biotin to the inactive apocarboxylases is catalysed by the enzyme HCS as illustrated in Figure 2.3.

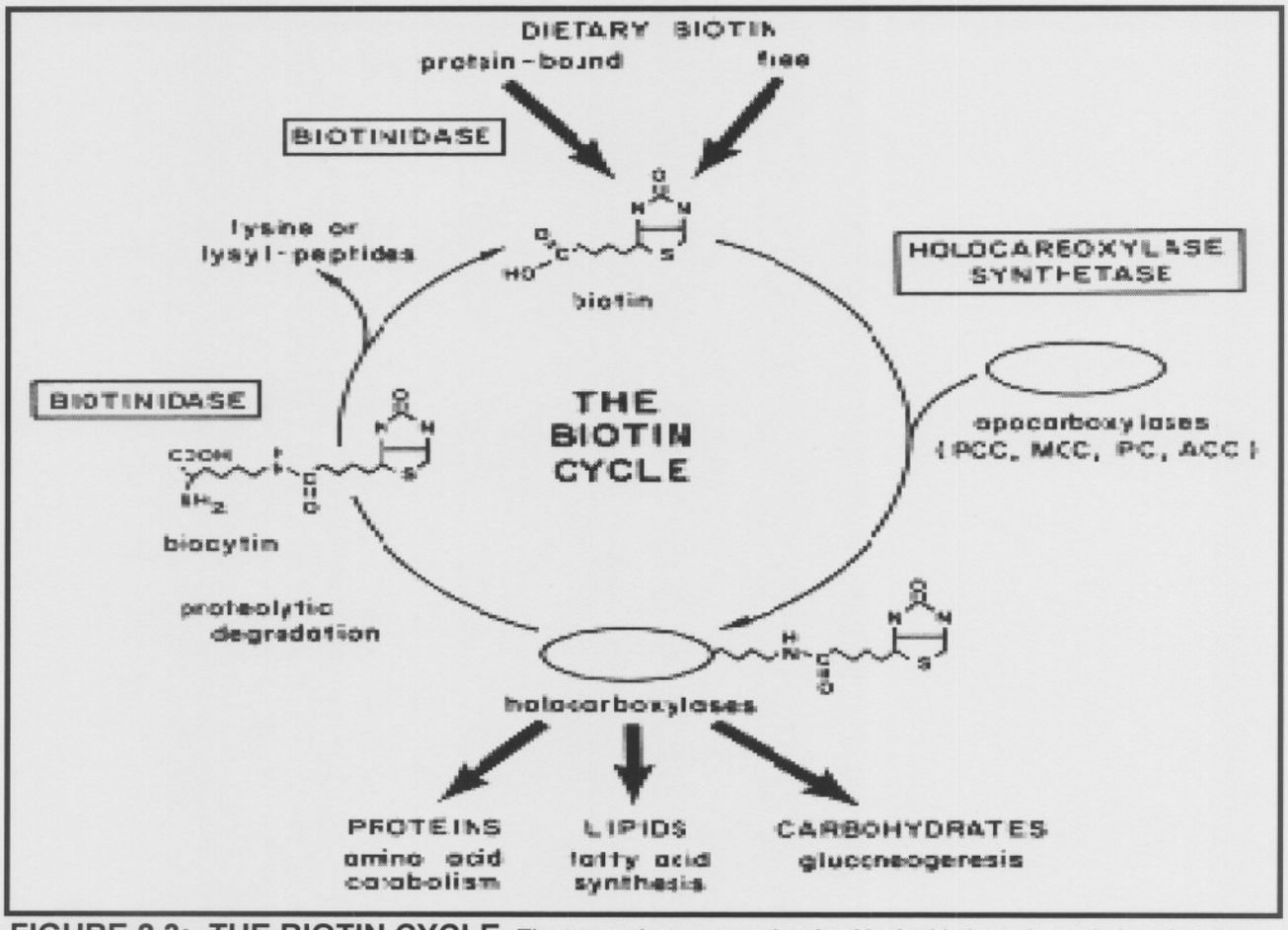


FIGURE 2.3: THE BIOTIN CYCLE. The two major enzymes involved in the biotin cycle are holocarboxylase synthetase (HCS) and biotinidase. MCC, 3-methylcrotonyl-CoA carboxylase; PCC, propionyl-CoA carboxylase; PC, Pyruvate carboxylase; ACC, acetyl-CoA carboxylase (Wolf, 2001)

Genetic defects of this enzyme lead to an inability to form active holocarboxylases resulting in MCD. Patients with MCD respond positively to biotin administration whereas those with MCC deficiency do not. However, contrasting reports regarding biotin administration and the influence on the clinical manifestation of MCC deficiency patients are documented (Sweetman and Williams, 2001; Baumgartner *et al.,* 2004; Friebel *et al.,* 2006). Generally patients suffering from MCC deficiency do not respond to biotin administration but evidence of biotin response are reported in patients who are heterozygous for MCCA-R385S as well as in the polymorphic variant MCCA-P464H (Baumgartner *et al.,* 2004). The first evidence of biotin responsive of MCC deficiency was documented in 2004 (Baumgartner *et al.,* 2004), where the first case of biotin responsive MCC deficiency was documented in 2006 (Friebel *et al.,* 2006).

2.4 TREATMENT OF 3-METHYLCROTONYL-CoA CARBOXYLASE DEFICIENCY

Acute episodes of MCC deficiency are usually treated with glucose and electrolytes to correct acidosis in the patient. The long-term treatment for MCC deficient patients is twofold: First, restriction of dietary protein to 0,750 – 2,00 protein (g) / body weight (kg) / day (d) to control the levels of the essential amino acid, leucine, and second, supplementation with glycine and/or carnitine to enhance detoxification ability of the accumulating toxic metabolites (Gibson *et al.,* 1994, Holzinger *et al.,* 2001). Although very few reports on combination therapy with glycine and carnitine have been published, it seems that the glycine and carnitine detoxification systems are complementary to each other rather than competitive (Gitzelmann *et al.,* 1987; Tsai *et al.,* 1989; Rolland *et al.,* 1991; Rutledge *et al.,* 1995). Case studies illustrated the importance of treatment of IEMs, especially in those patients with an early onset. It is important to note that irregular treatment causes neurological damage in patients (Lehnert *et al.,* 1996).

2.4.1 Detoxification therapy for MCC

Detoxification can be divided into two phases (Figure 2.4). Phase I reactions include oxidation, reduction and hydrolyses which are usually used to detoxify xenobiotic substances. Phase II detoxification includes the conjugation of xenobiotic and endogenous toxins to facilitate their urinary excretion as a soluble conjugated metabolite (Jakoby, 1980; Jakoby, 1981; Jakoby, 1990; Grant, 1991).

In the case of IEMs such as MCC deficiency, the rapid accumulation of metabolites exceeds the rate of detoxification. Therefore, the detoxification systems become overloaded and toxic endogenous metabolites accumulate. In general, treatment of patients with IEMs endeavors to enhance detoxification. The major detoxification systems operational in MCC deficiency are the phase II glycine and carnitine conjugation detoxification pathways. Due to exhaustion of these

systems, a secondary glycine and carnitine deficiency arises. Treatment with glycine doubles the excretion of 3-methylcrotonylglycine, with the maximum effect at 175 mg glycine/kg/day. L-carnitine (75 -100 mg/kg/d) should be administered to keep this system functioning properly. The excretion of glycine and carnitine conjugates increases significantly after glycine and carnitine therapy of MCC deficient patients (Gitzelmann *et al.,* 1987; Tsai *et al.,* 1989; Rolland *et al.,* 1991; Rutledge *et al.,* 1995; Baumgartner *et al.,* 2003).

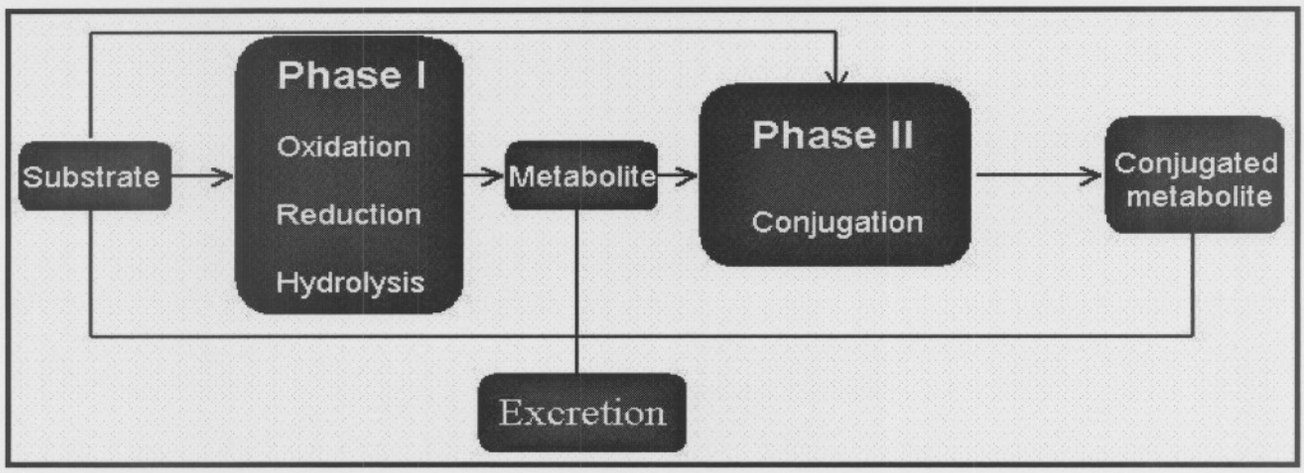
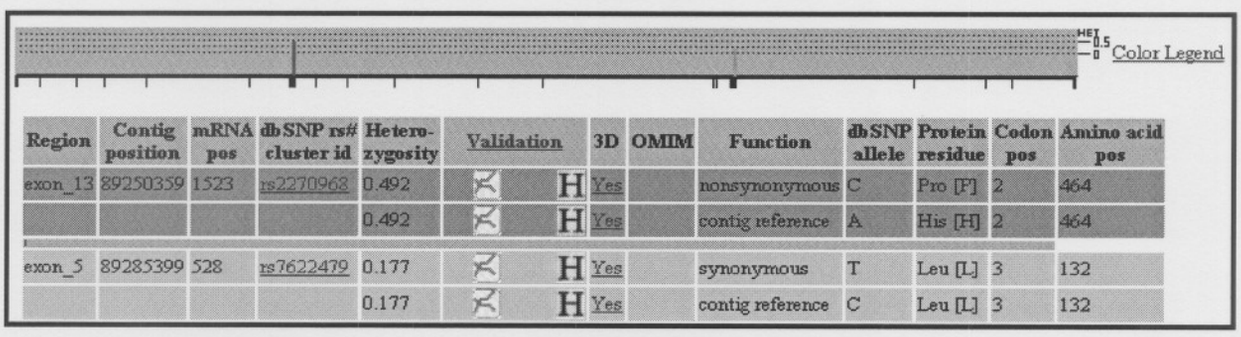


FIGURE 2.4: PHASE I AND PHASE II DETOXIFICATION OF THE HUMAN BODY
(Jakoby 1980, Jakoby, 1990, Grant 1991).

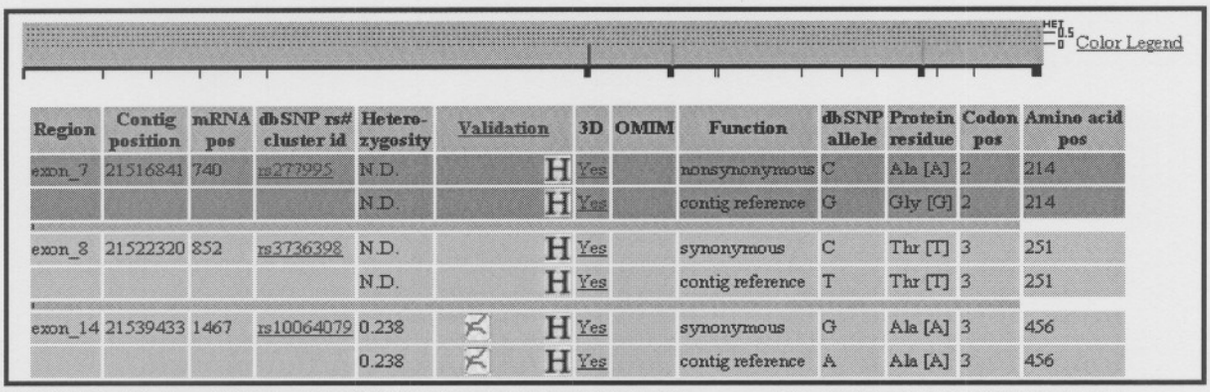
2.5 CHARACTERISTICS OF 3-METHYLCROTONYL-CoA CARBOXYLASE

MCC participates in the fourth step of the catabolism of the branched chain amino acid, leucine. It is a heteromeric biotin-dependent carboxylase that is predominantly localized to the inner membrane of the mitochondria and is expressed in mitochondria-rich organs, such as heart muscle, skeletal muscle, kidney and liver (Lehnert *et al.,* 1996; Obata *et al.,* 2001). The enzyme has an alpha subunit, *mccA,* and a beta subunit, *mccB.* These subunits are synthesised in the cell cytoplasm and are transported into the mitochondria by means of a signal peptide (Obata *et al.,* 2001).



Region	Contig position	mRNA pos	dbSNP rs# cluster id	Hetero-zygosity	Validation	3D	OMIM	Function	dbSNP allele	Protein residue	Codon pos	Amino acid pos
exon_13	89250359	1523	rs2270968	0.492	H	Yes		nonsynonymous	C	Pro [P]	2	464
				0.492	H	Yes		contig reference	A	His [H]	2	464
exon_5	89285399	528	rs7622479	0.177	H	Yes		synonymous	T	Leu [L]	3	132
				0.177	H	Yes		contig reference	C	Leu [L]	3	132

FIGURE 2.5: SNPs ASSOCIATED WITH GENE *mccA* LISTED IN GENBANK



Region	Contig position	mRNA pos	dbSNP rs# cluster id	Hetero-zygosity	Validation	3D	OMIM	Function	dbSNP allele	Protein residue	Codon pos	Amino acid pos
exon_7	21516841	740	rs277995	N.D.	H	Yes		nonsynonymous	C	Ala [A]	2	214
				N.D.	H	Yes		contig reference	G	Gly [G]	2	214
exon_8	21522320	852	rs3736398	N.D.	H	Yes		synonymous	C	Thr [T]	3	251
				N.D.	H	Yes		contig reference	T	Thr [T]	3	251
exon_14	21539433	1467	rs10064079	0.238	H	Yes		synonymous	G	Ala [A]	3	456
				0.238	H	Yes		contig reference	A	Ala [A]	3	456

FIGURE 2.6: SNPs ASSOCIATED WITH GENE *mccB* LISTED IN GENBANK

The biotin-dependent carboxylase family consists of four members, namely MCC (EC 6.4.1.4), propionyl-CoA carboxylase (PCC, EC 6.4.1.3); pyruvate carboxylase (PC, EC 6.4.1.1) and acetyl-CoA carboxylase (ACC, EC 6.4.1.2). PCC, MCC and PC function in the mitochondria whereas acetyl-CoA carboxylase functions in the cytosol (Holzinger *et al.,* 2001; Desviat *et al.,* 2003). Members of the carboxylase family have three structurally conserved functional domains. The biotin carboxyl carrier domain (A-M-K-M), where biotin is covalently attached to the ε-amino group of lysine. The biotin carboxylation domain (G-G-G-G-K-G-M-R-I-V), which catalyse the carboxylation of biotin and the carboxyl-tranferase domain (R-F-T-E-F-K-A-F-Y-G-D-T-L-V-T-G-F-A-R-I-F-G-Y-P-V-G-I-V-G), which catalyse the transfer of a carboxyl group from carboxybiotin to the organic substrate specific for each carboxylase **(**Samols *et al.,* 1988; Obata *et al.,* 2001**).**

The enzyme reaction catalysed by MCC illustrated in Figure 2.7. This reaction requires adenosine triphosphate (ATP), magnesium (Mg^{2+}), bicarbonate (HCO_3^-) and 3-methylcrotonyl-CoA as substrate to form the product trans-3-methylglutaconyl-CoA. The reaction is reversible. MCC is activated four to fivefold by the presence of potassium (K^+) and ammonium (NH_4^+). It is believed that 2-methylcrotonyl-CoA (Tiglyl-CoA) is also a substrate for MCC because most patients with disorders of the isoleucine catabolism in whom tiglyl-CoA accumulate, excrete (*E*)-2-methylglutaconic acid (Hector *et al.,* 1980; Sweetman and Williams, 2001).

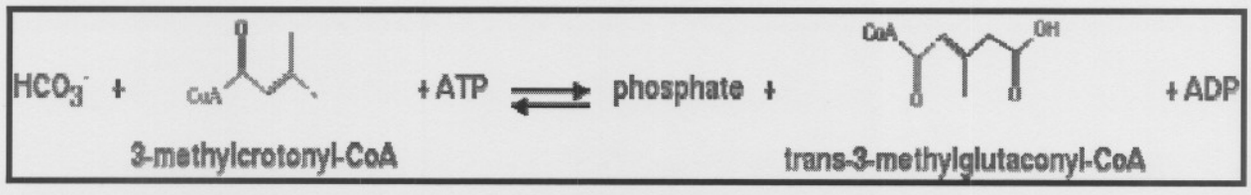


FIGURE 2.7: 3-METHYLCROTONYL-CoA CARBOXYLASE REACTION. HCO_3^-, bicarbonate; ATP, adenine triphosphate and ADP, adenine diphosphate; (Romero *et al.,* 2003).

The activity of MCC is usually assayed in fibroblast homogenates, lymphocytes (Beemer *et al.,* 1982) or cultured skin fibroblasts (Suormala *et al.,* 1985; Sweetman and Williams, 2001). MCC activity in cultured fibroblasts obtained from MCC deficient patients is usually less than 10% of the mean control value for healthy individuals (Lehnert *et al.,* 1996; Wiesmann *et al.,* 1998; Baumgartner *et al.,* 2001; Baumgartner *et al.,* 2004). There is no correlation between the level of residual enzyme activity and the severity of the clinical symptoms of this disease (Baumgartner *et al.,* 2001).

2.6 GENETIC BASIS OF 3-METHYLCROTONYL-CoA CARBOXYLSASE DEFICIENCY

The gene coding for the alpha subunit *mccA* is located on chromosome 3q25-27 (D3S1553-D3S1580) and consists of 19 exons. One hundred and ninety-five (195) single nucleotide polymorphisms (SNPs) have been identified for the subunit (*mccA*). Only two of these are in the coding region (Figure 2.5, GenBank, 2005). The open reading frame (ORF) of *mccA* (MCCA) is 2175 bases where the A of ATG is one (1) and TAA is the stop codon for translation, with a translation product of 725 amino acid residues. The gene encoding the smaller beta subunit *mccB* is located on chromosome 5q12-q13.1 (D5S637-D5S1977) and consists of 17 exons. For *mccB* 206 SNPs have been identified from which only three are present in the coding region (Figure 2.6, GenBank, 2005). The *mccB* ORF is 1685 nucleotides long and follows the same convention as in the case of *mccA*. The 1685 bases specify a protein consisting of 565 amino acid residues (Baumgartner *et al.,* 2000).

The phenotypic presentation of an inherited disease involves the expression of a specific gene. To understand the manifestation of such a disease, it is necessary to have a good understanding of the genotype (Scriver, 2004). In the case of MCC deficiency, several groups have reported the cloning and mutation analyses of both *mccA* and *mccB,* but no phenotype-genotype correlation could be made (Baumgartner *et al.,* 2001; Gallardo *et al.,* 2001; Holzinger *et al.,* 2001, Dantas *et al.,* 2005; Friebel *et al.,* 2006.

The most recent reports regarding MCC deficiency are those of Baumgartner *et al.* (2001), Gallardo *et al.* (2001), Holzinger *et al.* (2001), Dantas *et al.* (2005) and Friebel *et al.* (2006) Some patients were identified after presenting with clinical symptoms and others were identified by NBS programmes. The enzyme activity of the identified MCC mutations was assayed in cultured fibroblasts obtained form the patients and found to be less than 10 % of the control values (Baumgartner *et al.,* 2001; Gallardo *et al.,* 2001; Holzinger *et al.,* 2001, Dantas *et al.,* 2005). Table 2.2 and Table 2.3 list the documented mutations for *mccA* and *mccB* respectively. The shade

rows highlighted the mutations of of interest. The nucleic acid change, the amino acid involved, clinical severity and ethnic group of the affected patients are also given in these tables. The recorded frameshift mutations splice mutations and nonsense mutations result in truncated proteins lacking functionally important domains, whereas missense mutations lead to a possible change in amino acid (Baumgartner *et al.,* 2001; Gallardo *et al.,* 2001; Holzinger *et al.,* 2001; Dantas *et al.,* 2005).

TABLE 2.2: Summary of the reported mutations within *mccA*

Mutation	Exon	Nucleotide change	Clinical outcome	Population group	*Reference*
L132L synonymous SNP	5	396T→C	-	-	GenBank, Ensembl
E134K Missense	5	400G→A	-	German	Dantas *et al.*, 2005
S187P Missense	6	559T→C	-	German	Dantas *et al.*, 2005
R232W Missense	7	694C→T	-	American	Dantas *et al.*, 2005
G214lfsX5 Frameshift	7	640_641del GG	-	-	Dantas *et al.*, 2005
A291V Missense	8	872C→T	-	Australian	Dantas *et al.*, 2005
A289V	8	866C→T	Mild	American	Baumgartner *et al.*, 2001
M325R	10	974T→C	-	USA	Gallardo *et al.*, 2001
Q421AfsX10 Frameshift	11	1263dupG	Mild	Swedish	Dantas *et al.*, 2005
R385S Missense	11	1155A→C	Severe	German	Baumgartner *et al.*, 2001; Dantas *et al.*, 2005
R385S	11	1155A→C	Severe	USA	Gallardo *et al.*, 2001
Q421fs(+1)	11	1264insG	Mild	Swedish/ American	Baumgartner *et al.*, 2001
L437P	12	1310T→C	Severe	Argentine	Baumgartner *et al.*, 2001
C509X Nonsense	13	1527C→A	Severe	Turkish	Dantas *et al.*, 2005
C509SfsX38 Frameshift	13	1526_1257delG	-	American	Dantas *et al.*, 2005
D5532H	13	1594G→C	Severe	Turkish	Baumgartner *et al.*, 2001
S535F	14	1604C→T	-	-	Holzinger *et al.*, 2001
E644X Nonsense	17	1930G→T Skip exon 17	-	American	Dantas *et al.*, 2005
V697SfsX19 Frameshift	19	2088dupA	-	American	Dantas *et al.*, 2005
V694X	19	del2079A	-	-	Holzinger *et al.*, 2001

Compiled from Baumgartner *et al.,* 2001; Gallardo *et al.,* 2001; Holzinger *et al.,*2001; Danas *et al.,* 2005

TABLE 2.3: Summary of the reported mutations within *mccB*

Mutation	Exon	Nucleotide change	Clinical outcome	Ethnic group	Reference
Q43X Nonsense	1	127C→T	Mild	Arabian	Dantas *et al.*, 2005
R72X Nonsense	3	214C→T	-	Turkish	Dantas *et al.*, 2005
E99Q Missense	4	295G→C	Severe / Mild	Turkish / Amish	Baumgartner *et al.*, 2001; Dantas *et al.*, 2005; Holzinger *et al.*, 2001
C167R Missense	5	499T→C	-	Spain	Gallardo *et al.*, 2001
R155Q Missense	5	464G→A	Mild	Vietnamese	Baumgartner *et al.*, 2001
R155W Missense	5	463C→T	Mild	Brazilian	Dantas *et al.*, 2005
R155Q Missense	5	464G→A	-	Turkish	Dantas *et al.*, 2005
Q157X Nonsense	5	469C→T	-	Turkish	Dantas *et al.*, 2005
T139_G143→RWVPGEfsX35 Frame shift	5	416_427del12ins16	-	Turkish	Dantas *et al.*, 2005
In5ac-1G→A Splice site		In5ac-1G→A	Mild	Dutch	Baumgartner *et al.*, 2001
D172fs Frame shift	6	517insT	-	USA	Gallardo *et al.*, 2001
R193C Missense	6	577C→T	Mild	Dutch	Baumgartner *et al.*, 2001
S173L Missense	6	518C→T	Severe	Turkish	Baumgartner *et al.*, 2001
H190Y Missense	6	568C→T	Mild	Turkish	Dantas *et al.*, 2005
S173FfsX25 Frame shift	6	517dupT	Mild	Swiss / Mennonite Swiss American	Baumgartner *et al.*, 2001 Dantas *et al.*, 2005
A218T Missense	7	652G→A	-	Argentina	Gallardo *et al.*, 2001
R268T Splice	8	803G→C	--	Turkish-	Holzinger *et al.*, 2001; Dantas *et al.*, 2005
H282R Missense	9	845A→G	-	Italian	Dantas *et al.*, 2005
R332X Nonsense	10	994C→T	Mild	Swiss	Dantas *et al.*, 2005
P310R Missense	10	929C→G	Mild	Vietnamese Australian	Baumgartner *et al.*, 2001 Dantas *et al.*, 2005
G352R + V334_G358delinsKFFMKYFL RLDLNSYNSTWQH Splice	11	1054G→A	Mild	Turkish	Dantas *et al.*, 2005
V339M Missense	11	1015G→A	Severe	Turkish	Baumgartner *et al.*, 2001
V375F Missense	12	1123G→T	-	American	Dantas *et al.*, 2005
I437V Missense	14	1309A→G	Mild	Dutch	Baumgartner *et al.*, 2001
A456V Missense	14	1367C→T	-	Taiwanese	Dantas *et al.*, 2005
X564QLE Add aa at 3' terminus	17	1690T→C	-	Australia	Dantas *et al.*, 2005

Compiled from Baumgartner *et al.*, 2001; Gallardo *et al.*, 2001; Holzinger *et al.*, 2001; Danas *et al.*, 2005

2.6.1 Mutations of *mccA*

The MCCA-R385S mutation appears to be the most frequent one found in MCC deficient patients and is associated with severe clinical symptoms. It seems that patients of German origin are more often affected (Baumgartner *et al.,* 2001 and Gallardo *et al.,* 2001). Expression studies showed that the MCCA-S385 is catalytically inactive Baumgartner (2001). It has been suggested that MCCA-R385S is a dominant negative allele and that is biotin responsive in vivo (Baumgartner *et al.,* 2004; Baumgartner, 2005). A very recent paper on MCC deficiency (Friebel *et al.,* 2006) reported a nine-year-old boy with severe psychomotor retardation who developed infantile spasms at the age of three weeks. Biochemical findings indicated MCC deficiency in cultured fibroblasts. Molecular studies showed a heterozygote missense mutation, MCCA-R385S. Biotin therapy led to a dramatic decrease in the frequency of seizures, and a near normalisation of organic aciduria.

Four missense mutations have been identified that are associated with the highly conserved biotin carboxylation domain. These mutations are E134K, S187P, R232W and A291V. In expression studies, A291V was associated with a 26 % reduction of MCC activity compared to the normal value (Dantas *et al.,* 2005).

2.6.2 Mutations and *mccB*

There appears to be 25 mutations in *mccB* in the exons or open reading frame (coding region) of deficient *mccB*'s (Baumgartner *et al.,* 2001, Gallardo *et al.,* 2001, Holzinger *et al.,* 2001 and Dantas *et al.,* 2005). These mutations include missense, nonsense and frameshift mutations. A compilation of the described mutations involving the coding region is listed in Table 2.3.

No MCC enzyme activity was measured in the presence of the mutations R155Q, P310R, S173L, E99Q, MCCB-V339M and R193C had some residual activity, about 4% of the experimental control value. The alpha subunit seems to be less stable when the beta subunit is absent or defective (Baumgartner *et al.,* 2001). The missense mutation 803G→C; R268T, is positioned within the carboxylase domain. Therefore, the mutation could impair MCC function or it could alter splicing significantly, since the mutation affects the conserved most 3'-positioned guanine residue of exon 8 (Holzinger *et al.,* 2001).

Two observations underscore the difficulty to correlate genotype with phenotype. First, the finding that almost every patient has a unique genotype with no prevalent mutation for either *mccA* or *mccB* suggested that factors other than the genotype at the MCCA and MCCB loci must have a major influence on the phenotype of MCC deficiency. Second, when different patients have the

same homozygous E99Q mutation, they have very different clinical symptoms (Baumgartner *et al.*, 2001; Gallardo *et al.*, 2001).

Comparing the molecular basis of MCC deficiency to PCC deficiency, it is clear that PCC deficient patients have no prevalence for a specific mutation. Some correlations have been found in PCC deficient patients. Mutations categorized as null alleles, which include nonsense, frameshift, deletions, insertions and splice site mutations are associated with the most severe phenotypes. Missense mutations retaining partial activity *in vitro* are associated with a mild phenotype (Desviat *et al.*, 2003).

2.7 NEWBORN SCREENING PROGRAMMES AND PRENATAL DIAGNOSIS

There are three reasons to perform genetic screening: (1) for early diagnosis and treatment; (2) for the identification of carriers to improve reproductive counselling; and (3), to gather information that would contribute to better knowledge and understanding of a specific condition (Scriver, 2006).

The concept of NBS programmes was first introduced by Robert Guthrie in the 1960s. The diagnosis of phenyl ketonuria (PKU) in the mentally retarded niece of his wife stimulated his interest in the underlying cause and ways to prevent mental retardation. He developed a bacterial inhibition assay for phenylalanine (Guthrie and Susi, 1963). Later he also developed bacterial inhibition assays for other metabolites so that additional metabolic disorders could be detected. Further developments followed, such as screening for congenital hypothyroidism and sickle-cell disease. The most recent of these developments is the use of mass spectrometry (MS) Guthrie 1968. MS makes it possible to detect a much wider spectrum of metabolic disorders in a single assay (Qu *et al.*, 1991; Levy 1998; Levy and Albers 2000; Fearing and Levy 2003; Marsden *et al.*, 2006). New MS/MS methodologies are currently being evaluated. There is quite a variation of the number of IEMs that are screened for. The American Academy of Paediatrics has recommended a panel of 29 disorders that should be screened for in all States in the United States of America (USA). It is likely that in the future, NBS will be available to identify much more inborn errors of metabolism (Marsden *et al.*, 2006).

The overall frequency of metabolic disorders is considered to be 1:4000 newborn babies (Zytkovicz *et al.*, 2001; Chace *et al.*, 2002; Schulze *et al.*, 2003; Wilken *et al.*, 2003; Marsden *et al.*, 2006). The expansion of NBS programmes brought several challenges and issues to the fore. One of the important issues in expanded screening is the considerably larger number of cases of certain disorders that are detected. These disorders include medium chain acyl-CoA dehydrogenase deficiency (MCADD), very long chain acyl-CoA dehydrogenase deficiency (VLCADD), short chain

acyl-CoA dehydrogenase deficiency (SCADD), glutaric academia type I (GA-I), isovaleric academia (IVA), 3-methylcrotonyl-CoA carboxylase deficiency (MCC) and 3-ketothiolase deficiency (Marsden *et al.,* 2006)

Benefits of extensive NBS programmes include the: early identification of individuals with a treatable metabolic disorder; the identification of IEM cases that contribute to improve their genetic characterisation; the development of optimal treatment protocols and improved clinical outcomes. It is important for mothers to be screened simultaneously with their babies, since MCC was detected in several adult woman during mass screening of their newborn babies (Obata *et al.,* 2001). Mutation analyses should be done as a follow-up on samples presenting an abnormal metabolic profile. It can be performed on the DNA from a blood card sample of newborn. Because the clinical phenotype in some diseases such as MCC deficiency may not present until adulthood, long term follow-up is essential. Many disorders have hundreds of mutations and new ones will continue to arise. Therefore, secondary confirmatory testing is necessary to extend and improve genetic counselling (Marsden *et al.,* 2006).

MCC deficiency is currently the organic aciduria that is most frequently detected in mass spectrometry based screening programmes. The overall frequency is approximately 1 in 50,000 patients. Therefore, MCC deficiency is considered as an emerging disease. The incidence of MCC in South Africa and Africa as a whole is not known because of the absence of NBS programmes. In a first pilot study in South Africa that screened a diverse group of 12 000 individuals for IEMs, no cases of MCC deficiency were detected (Knoll personal communication). This result was not unexpected since the incidence of MCC is 1:50 000 (Baumgartner *et al.,* 2003).

A case report of 1984 illustrates the importance of early diagnosis of inborn errors of metabolism Bartlett *et al.,* 1984. Five children were born from a non-consanguineous marriage. Three died before the age of 3. The first pregnancy resulted in the birth of twins. One was stillborn and the other died 2 days after birth. The second pregnancy produced a healthy boy and the third a boy who died at 2 years. Subsequently the couple also had another child, a girl. She was born after a normal pregnancy and normal delivery, with no perinatal complications. However, she had repeated otitis media. At 22 months, she presented in a hypotonic, collapsed and unconscious state. Laboratory investigation showed hypoglycaemia, mild acidosis and gross neutrophilia. After several tests, doctors diagnosed MCC (Bartlett *et al.,* 1984). This case study is one of many case reports that illustrate the need for the diagnosis of inborn errors of metabolism in newborns. The introduction of NBS programmes worldwide contributes to the establishment of such early

diagnosis. Undoubtedly, NBS already significantly impact on mortality and morbidity in countless children.

2.8 PROBLEM STATEMENT AND AIMS

2.8.1 Problem statement

The introduction of MS/MS and the development of molecular methods are transforming diagnostics of IEMs to become significantly more sensitive and specific. The lack of genotype-phenotype correlation is one of many unresolved issues in IEMs (Lanpher *et al.,* 2006). Factors other than genotype that seem to have an impact on the clinical outcome of IEMs include environmental factors and the efficacy of detoxification of toxic metabolites that accumulate as a result of the enzyme defect. To date, it has not been possible to correlate the level of residual MCC enzyme activity and clinical presentation or determine a genotype-phenotype correlation. Important questions that remain to be answered include: "(1) Why do some patients develop symptoms while others, even in the same family, remain asymptomatic? (2) What are the variables (environment, modifying genes) determining the phenotype?" (Baumgartner *et al.,* 2003).

The introduction of tandem mass spectrometry based NBS programmes in Europe, North America and Australia has focused attention on MCC deficiency. It is the most frequent organic aciduria detected by NBS programmes. With a frequency of 1:50 000, it is considered as an emerging disease (Baumgartner *et al.,* 2001; Holzinger *et al.,* 2001). The incidence of MCC deficiency in Africa is not yet known. All that is known is that the metabolic unit of the School of Biochemistry of the North-West University identified one individual with from MCC deficiency through biochemical analyses of metabolites present in his urine and blood. The patient had high levels of detoxification metabolites. This observation, amongst others, has lead to the start of a research program that aims to investigate the role of the efficiency of detoxification in the phenotype of IEMs.

During the past five years (2001-2006) the molecular characterisation of MCC deficiency and other IEMs has progressed rapidly. For RSA to get to the forefront of the field, it is necessary to implement and develop the same molecular approaches to confirm, complement and extend the biochemical analyses of IEMs.

2.8.2 Aims

The aims of this study are to:

1. implement the appropriate techniques for molecular diagnosis and characterisation of MCC deficiency, and

2. sequence the coding region of the MCC genes *mccA* and *mccB* to determine which mutation(s) are present in the MCC deficient South African patient.

The study will also contribute to the development of genetic counselling that should be available to families of affected patients.

2.9 STRATEGY AND EXPERIMENTAL APPROACH

It is necessary to study the basis of metabolism, enzymology and the incidence of MCC in NBS programmes in RSA. This must include the molecular characteristics of the RSA variant of MCC deficiency in order to characterize the entire molecular and biochemical profile of MCC deficiency. Implementation of the molecular characterisation of MCC deficiency in South Africa is the focus of this study. Two parallel approaches will be followed e.g. on the genomic DNA level and on the level of mature messenger ribonucleic acid mRNA transcript (Figure 2.8).

Cultured skin fibroblast cells of a MCC non-deficient individual will serve as starting material for the standardisation of fibroblast based methods. Blood from control individuals will be used for the standardisation of the buffy coat methods. Thereafter patient analyses of the implemented methods will follow.

The advantage of the ribonucleic acid (RNA) approach is that the entire sequence of the coding regions of the two subunits *mccA* (19 exons) and *mccB* (17 exons) can be determined. This requires the isolation of total RNA from cultured skin fibroblasts and/or white blood cells. The isolated total RNA will then serve as template for reverse transcriptase (RT) or cDNA synthesis. The entire open reading frames for both *mccA* and *mccB* will be amplified by using *mccA* and *mccB* specific primers. Computer sequence analyses programmes such as *Chromas* and *Vector NTI* will be used to analyse sequence data in order to identify variation in nucleotide, mutations and SNPs.

In this study, the genomic approach will serve as a verification of mutations identified on the mature mRNA transcript of both MCC genes. The exons of interest related to published mutation analyses of MCC will be used as the basis. The South African MCC deficient patient is a Caucasian of German origin. Therefore, it is likely that the mutation(s) could be among those detected in other Caucasians, especially Germans. For *mccA*, exon 8 and 11 will be included and for *mccB* exons 5 and 6 will be included. The genomic approach includes the isolation of DNA, while only specific exons of interest will be amplified. After the amplification of these exons, direct sequencing and mutation analyses will be done. The flow diagram (Figure 2.8) illustrates the experimental layout of this investigation. The characterisation of MCC deficiency in RSA involves four legs i.e.

enzymology, the incidence of MCC in NBS programmes, molecular biology and metabolism. The shaded region represents the experimental design of molecular biology. The pink block indicates the experimental steps accomplished in this study whereas the white block should still be done.

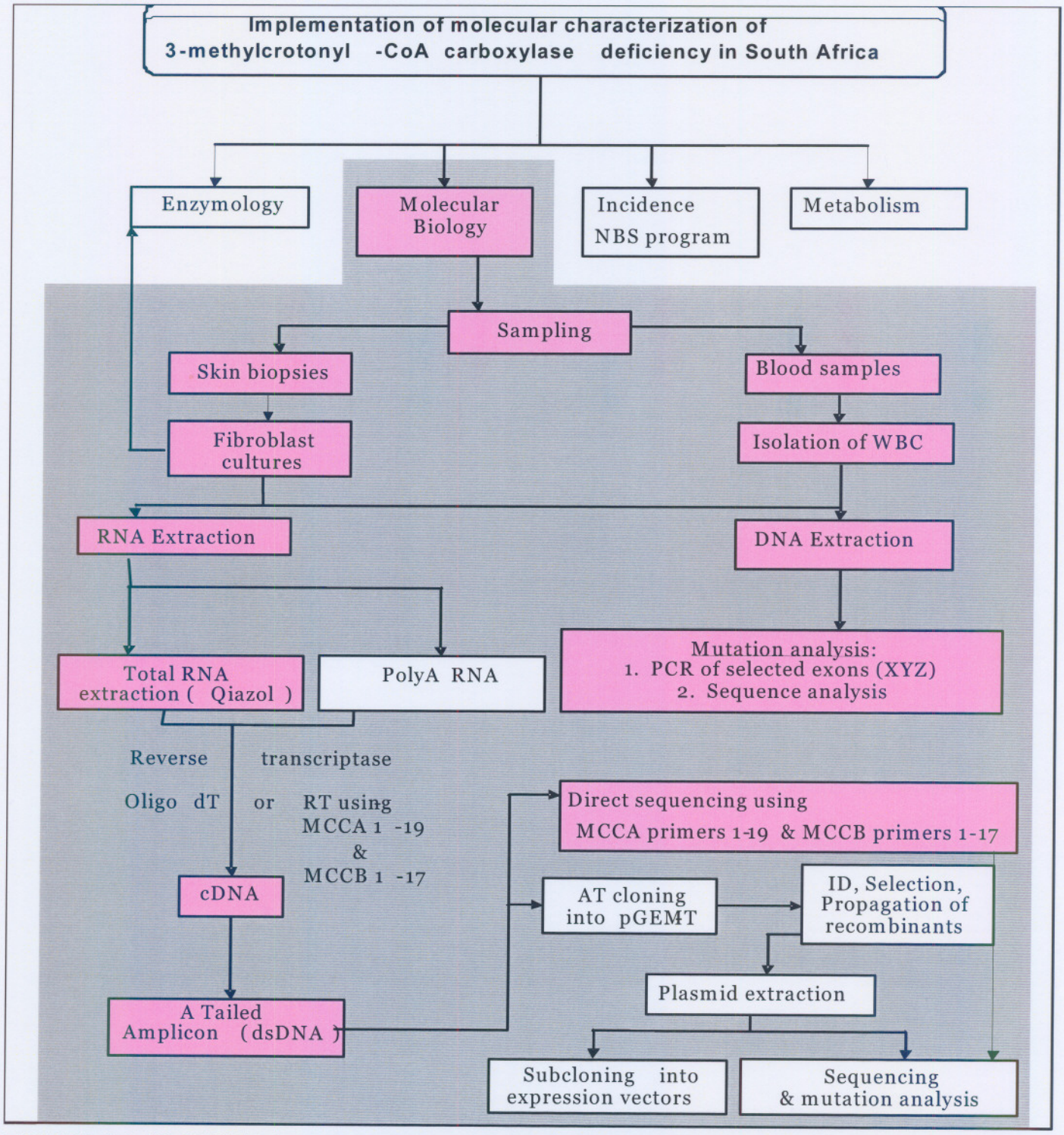


FIGURE 2.8: FLOW DIAGRAM OF THE EXPERIMENTAL APPROACH FOR THE IMPLEMENTATION OF THE MOLECULAR CHARACTERISATION OF 3-METHYLCROTONYL-CoA CARBOXYLASE DEFICIENCY IN SOUTH AFRICA. The pink block indicates the experimental steps accomplished in this study where the white block should still be done.

Chapter Three

Molecular characterisation of *mccA* and *mccB* mature mRNA transcripts

Molecular confirmation and detailed molecular characterisation of the biochemical diagnosis of metabolic diseases by mutation analyses has become important to gain more knowledge of these diseases. It also helps to establishing genotype-phenotype correlations and, where applicable, to initiate the appropriate therapy (Oliver, 2002).

Currently, MCC deficiency is the organic aciduria most frequently detected in MS based screening programmes in North America (Gibson *et al.,* 1998; Naylor and Chace, 1999; Smith *et al.,* 2000), Europe (Roscher *et al.,* 2000) and Australia (Wilcken *et al.,* 2000). The expansion of MS/MS based NBS programmes brought a considerable larger number of MCC deficiency cases to the fore. Therefore, MCC deficiency is considered as an emerging disease (Gibson *et al.,* 1998; Naylor and Chace, 1999; Smith *et al.,* 2000; Roscher *et al.,* 2000; Wilcken *et al.,* 2000). The overall frequency is approximately 1 in 50,000 patients screened (Baumgartner *et al.,* 2003). Follow-up and genetic counselling is of the most important aspects to take cognisance of following the diagnosis of an IEM by NBS programmes (Hoff and Hoyt 2006). The development of molecular methods to expand NBS programmes is transforming diagnostics of IEMs to become significantly more sensitive and specific.

Several groups are currently working on various aspects of this emerging disease. Their focus is on the molecular basis of MCC deficiency (Baumgartner *et al.,* 2001; Gallardo *et al.,* 2001; Holzinger *et al.,* 2001; Dantas *et al.,* 2005; Stadler*et al.,* 2006). The focus of this study was to attempt to elucidate the molecular basis of a South African case of MCC deficiency. The associated genes *mccA* and *mccB* are well characterized. The α-subunit, *mccA*, is located on chromosome 3q25-27 (D3S1553-D3S1580).The ORF consists of 2275 bases and 19 exons that code for a protein of 725 amino acids. The smaller β-subunit, *mccB*, is located on chromosome 5, 5q12-q13.1 (D5S637-D5S1977). The ORF consists of 1824 bases and 17 exons that code for a 563 amino acid protein. Mutations described in these genes are listed in Tables 2.2 and 2.3. Up to now no correlation of residual enzyme activity could be made to the clinical symptoms of the patient (Baumgartner *et al.,* 2001).

In summary, the following is already well-established in European laboratories and should be implemented in our laboratory to operate a basic molecular characterisation program in order to develop a well functioning extended molecular characterisation program. In general, a two-pronged approched is applied i.e. the amplification of the OFRs of the associated genes (*mccA* and *mccB*) and the amplification of the corresponding gDNA (genomic DNA) fragments (exons). These exons are amplified using flanking intronic primers. The primers used for RT-PCR are located in the non-coding regions of each gene. This ensures that the required fragment is amplified. The isolation of nucleic acids is done using commercial available kits. In most cases the Flexigene® gDNA isolation kit was used for the isolation of gDNA and Qiazol® lyses reagent is used for the isolation of total RNA. Amplification of gDNA fragments from synthesised cDNA and gDNA for mutation analyses is well documented. One-step RT-PCR is more frequently used in molecular studies of MCC deficiency. (Baumgartner *et al.,* 2001; Gallardo *et al.,* 2001; Holzinger *et al.,* 2001 and Dantas *et al.,* 2005). Direct sequencing is performed to determine the nucleic acid sequence of the particular amplicon. Mutation analyses using computer software confirms the findings.

In this chapter the molecular techniques are described for the amplification of the genes coding for *mccA* and *mccB* of a control individual (not MCC deficient) which is needed to study and identify mutations present in *mccA* and/or *mccB* of the MCC deficient South African patient. These genes (*mccA* and *mccB*) are well described in the literature (Baumgartner *et al.,* 2001; Gallardo *et al.,* 2001; Holzinger *et al.,* 2001 and Dantas *et al.,* 2005, Stadler *et al.,* 2006).

Mutations described for the *mccA* subunit which consists of 725 amino acids are listed in Table 2.2. The beta subunit is smaller and has 563 amino acids. Mutations described for *mccB* are listed in Table 2.3. Mutation analyses of MCC deficiency described in the literature is done on cDNA from mRNA as well as on genomic level. Either total RNA or mRNA from cultured fibroblast cells is used. Gene amplification is achieved as a one step RT-PCR using primers in the 5' UTR and 3'-UTR of *mccA* and *mccB* respectively. The PCR product is then gel purified and directly sequenced. To confirm mutations identified in RT-PCR products, amplification of genomic fragments containing the corresponding exon, using flanking intronic primers are done. These PCR amplicons are sequenced directly (see Chapter 4) (Baumgartner *et al.,* 2001; Gallardo *et al.,* 2001; Holzinger *et al.,* 2001 and Dantas *et al.,* 2005).

In Chapters three and four the same two-pronged approach for the molecular characterisation of MCC deficiency, as described in Chapter 2 paragraph 2.8.2 figure2.8, will be implemented. This chapter focuses on using RNA as departure point and generally for cDNA amplification, sequencing

and mutation analyses of the *mccA* and *mccB* transcripts (Baumgartner *et al.*, 2001). An outline of the experimental procedures described in this chapter is depicted in Figure 3.1.

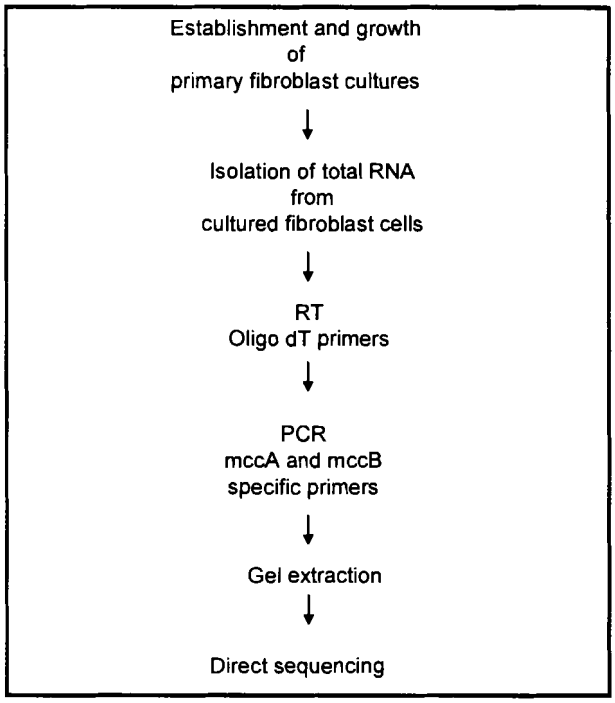


FIGURE 3.1: EXPERIMENTAL APPROACH FOR THE MOLECULAR CHARACTERISATION OF THE *mccA* AND *mccB* MATURE mRNA TRANSCRIPTS

3.1 MATERIALS AND METHODS

3.1.1 Biological samples

This project was approved by the Ethics Committee of the North-West University (05M14). Two different skin fibroblast cultures were used. One culture was from an infant who did not have MCC deficiency (CFC). The other skin biopsy was from an individual (HGS) who have MCC deficiency. Whole blood from three healthy adults, were collected in Heparin coated tubes. Buffy coats were isolated. Protocols followed in this study were done according to the specifications included with the respective commercial kits used, or according to published protocols as indicated. Modifications to these protocols are indicated in the text.

3.1.1.1 Fibroblast cell cultures

Primary fibroblast cultures were from skin biopsies taken and established at GENEPATH, located at the Jakaranda Hospital, Sunnyside, Pretoria, South Africa. The culture from the MCC non-deficeint individual originated from a skin biopsy taken from a one-month-old baby boy (CFC) suspected to have gangliosidosis type 1 (see Appendix D). These cells were used to implement techniques for the molecular characterisation of MCC deficiency. The other primary fibroblast

culture was from a skin biopsy of an adult patient (HGS) biochemically diagnosed with MCC deficiency.

The fibroblast cell cultures were received from GENEPATH in 25 cm^2 flasks and propagated further in either Amniochrome system II® medium (cat no.BE12-756F, Cambrex) supplemented with 0,125 mg/ml Penicillin/Streptomycin (cat no.17-602E, Cambrex) and 0,256 mg/ml Gentamycin (cat no.17-5182, Cambrex) or in DMEM (cat no.41966-029, Gibco) supplemented with 10% fetal bovine serum (FBS) (cat no. 10108-165, Gibco) and 0,1mg/ml Penicillin/Streptomycin (cat no.17-602E, Cambrex) and incubated in 5% CO_2 at 37 °C. The medium was changed twice a week by decanting the old medium and washing the excess medium and dead cells off with sterile phosphate buffered saline (PBS, cat no. BR0014G, Oxoid). When the cells reached 80 – 100% confluency, the medium was removed and the cells were washed with 1 X PBS. The cells were then incubated a few minutes with 1 X trypsin-EDTA (ethylenediaminetetraacetic acid) (cat no. 15400-054, GIBCO) until they detached from the flask. Fresh growth medium was added to inhibit trypsin activity. The cells were split in a 1:3 and 1:4 ratio for 25 cm^2 and 75 cm^2 flasks (cat no.90025 and 90075, TPP) respectively. Fresh medium was added to each flask to a volume of 5 ml for the 25 cm^2 and 15 ml for the 75 cm^2 flask. The flasks were then incubated at 37 °C and 5% CO_2 for 24 hours. Fresh medium was added once and cultures were incubated until 80 – 100 % confluency was reached.

Cells were used for DNA and RNA isolation when 80% – 100% confluency was reached (1-3 x 10^6 cells per 25 cm^2). Cells were harvested, washed with PBS and stored as a semi-dry pellet at -80°C in 1,5 ml tubes until needed for gDNA isolation. Cells that were used for total RNA isolation were stored at -80 °C in 1,5 ml tubes containing 200 µl RNA*later*™ (cat no. 7020, Ambion). RNA*later*™ is a reagent that stabilizes RNA and prevents the RNA from degradation.

3.1.1.2 Stabilisation of RNA with RNA*later*™

Preservation of RNA in biological material contributes to the success of the downstream applications. The quantity of RNA isolated from the biological sample depends on the quality of the preservation of the RNA in the starting materials. RNA*later*™ technology is designed for stabilization and protection of cellular RNA in animal tissues and cell cultures (RNA*later*™ Handbook, 2003 Qiagen).

3.1.2 Isolation of total RNA

For the isolation of total RNA, commercial kits were used according to the instructions of the manufacturers (Qiagen), i.e. RNeasy® total RNA isolation kit and the Qiazol® reagent. The step by step laboratory protocols for these methods are presented in Appendix I.

3.1.2.1 Isolation of total RNA using the Qiazol®

Total RNA isolation was done using Qiazol® lyses reagent (cat no. 79306, Qiagen). Qiazol® is a monophasic solution that contains phenol and guanidine thiocyanate. This reagent is based on a single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform (AGPC) extraction as described by Chomczynzki and Sacchi (1986). It maintains the integrity of the RNA, while disrupting cells and dissolving cell components.

The denaturing solution, known as solution D (Chomczynzki and Sacchi, 1986) consists of 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7); 0,5 % sarcosyl; 0,1 M 2-mercaptoethanol. This solution can be stored for 1 month at room temperature. It is commercially available as Qiazol® lyses reagent. After the addition of this mixture to the cells, the samples were vigorously shaken and homogenized with a syringe through a 28 gauge needle. The homogenate was cooled on ice, whereafter it was centrifuged to form two layers. The RNA present in the upper aqueous phase was removed and isopropanol was added to precipitate the RNA. The volume of the aqueous phase obtained from the phase separation determines the amount of isopropanol needed. The ratio of aqueous phase to isopropanol is 2:1. The RNA pellet was then washed with 75 % ethanol, dried and dissolved in 0,5 % sodium dodecyl sulphate (SDS) at 65 °C for 10 min (Chomczynzki and Sacchi, 1986). For the Qiazol® reagent, RNA was dissolved in 50 µl RNase free water. The Qiazol® RNA extraction procedure is outlined in Figure 3.2.

3.1.2.2 Isolation of total RNA using the RNeasy® kit

RNeasy® (cat no. 74104) technology combines the selective binding properties of a silica-gel-based membrane with the speed of microspin technology. Biological samples are lysed and homogenized in the presence of a highly denaturing guanidine isothiocyanate (GITC)-containing buffer, which immediately inactivates RNases to ensure isolation of intact RNA. Ethanol is added to provide appropriate binding conditions, and the sample is then applied to an RNeasy® mini column where the total RNA binds to the membrane and contaminants are washed away. GITC-containing lyses buffer and ethanol create conditions that promote selective binding of RNA to the RNeasy® membrane. High-quality RNA is then eluted in 30- 50 µl RNase free water. This procedure provides enrichment for mRNA since most RNAs smaller than 200 nucleotides, such as 5.8S ribosomal

RNA (rRNA), 5S rRNA, and transport RNA (tRNAs), which together comprise 15–20% of total RNA, are selectively excluded. The RNeasy® RNA extraction method is outlined in Figure 3.3.

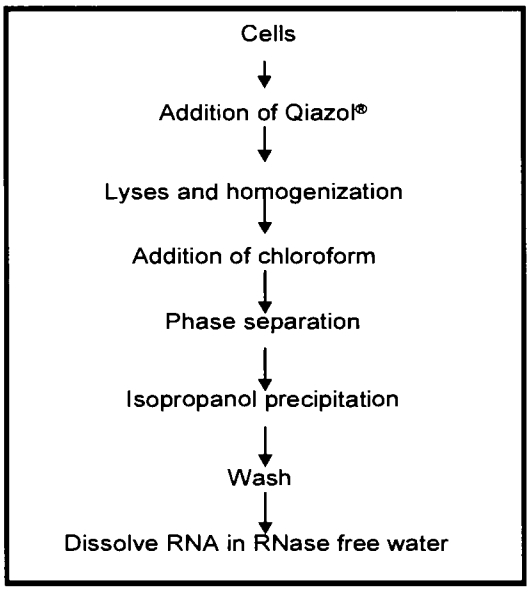


FIGURE 3.2: FLOW DIAGRAM OF THE BASIC QIAZOL® RNA EXTRACTION

Cultured human fibroblast cells from one 25 cm^2 flask (approximately 2 X 10^6 cells) were used for each extraction regardless the method used. As described in Section 3.1.1.1 the cells were harvested when 80 – 100% confluency was reached. Following centrifugation, 200 µl RNA*later*® was added to the cell pellets. The cells in RNA*later*® were stored at -70 °C until needed for RNA extraction. Isolated RNA was analysed by gel electrophoresis on a 1 % tris-acetic acid EDTA (TAE) agarose gel or a 1,2 % formaldehyde denaturing gel.

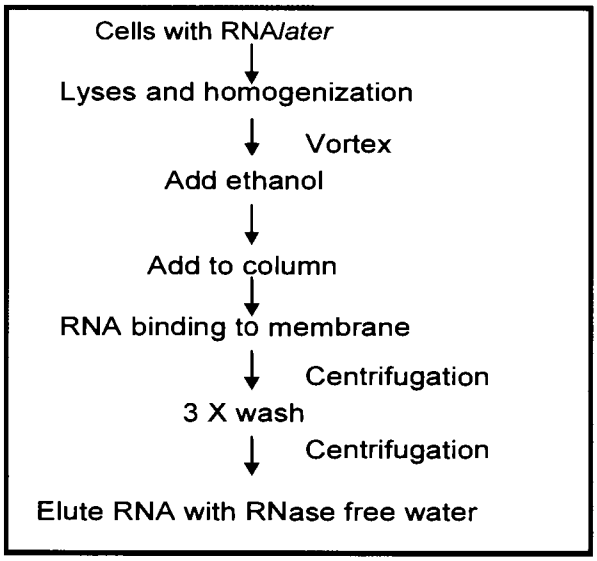


FIGURE 3.3: FLOW DIAGRAM OF THE BASIC RNEASY® METHOD

3.1.3 Quantification and characterisation of RNA

3.1.3.1 Spectrophotometric quantification

For a RNA solution, one optical density at 260 nm (OD_{260}) corresponds to 40 ng/µl. Spectrophotometric analyses was done with a NanoDrop® instrument (NanoDrop® ND1000) with which very small volumes of samples can be measured directly. Small sample volumes can be measured. The purity of DNA is often ascertained by a parameter commonly referred to as the A_{260}/A_{280} ratio. It is important to note that the A_{260}/A_{280} ratio is only an indication of purity rather than a precise answer. When RNA preparations are pure the A_{260}/A_{280} ratio is 2.0. The concentration of isolated total RNA can be calculated using equation 3.1. The automatic calculation uses the same parameters to determine the concentration of the isolated total RNA as those used in Equation 3.1. The total yield obtained from the isolation is calculated using Equation 3.2.

EQUATION 3.1: CALCULATION OF THE CONCENTRATION OF RNA FROM THE ABSORBANCE AT 260 nm

$$[\text{single-stranded RNA}] = A_{260} \times (40\ \text{ng}.\mu\text{l}^{-1} \times \text{dilution factor})$$

[RNA] = RNA concentration; A_{260} = absorbance of samples at 260 nm (Sambrook and Russell 2003).

EQUATION 3.2: CALCULATION OF THE YIELD OF RNA

$$\text{Total yield} = \text{concentration} \times \text{volume of sample in millilitres}$$

3.1.3.2 Gel electrophoresis

Agarose gel electrophoresis

For analytical purposes a 1 % TAE agarose gels with a final concentration of 0,5 µg/ml ethidium bromide was used. Unless otherwise indicated a 8 µl sample consisting of 5 µl PCR amplicon, gDNA or total RNA and 3 µl 6 X loading buffer was pipetted in a well (see Appendix G). The gels were run for 60 min at a constant voltage of 70 V. For preparative purposes, the samples for gel extraction consisted of 45 µl PCR product and 10 µl loading buffer. These were loaded in a preparative gel. Electrophoresis was for 60 min at 70 V using a Bio-Rad PowerPac Basic system. Agarose gels were visualized using UV transillumination of a gel-documentation system (Syngene ChemiGenius Bio-Imaging System) and GeneSnap software (Syngene, England).

Formaldehyde denaturing gel electrophosesis

Denaturing agarose gel electrophoresis and ethidium bromide staining was used to verify the integrity of the total RNA that was isolated. The respective 18S and 28S ribosomal RNA bands should appear as sharp bands in the stained gel and the 28S ribosomal RNA band should be present at an intensity approximately twice that of the 18S RNA (RNAeasy® Handbook, 2001). To estimate the integrity of RNA samples, 4 µl from each 50 µl sample preparation together with 1 µl RNA loading buffer (Appendix G) was loaded on a 1,2 % formaldehyde (FA) denaturing gel. The formaldehyde denaturing gels consisted of 1,2 % agarose (w/v), FA gel buffer with a final concentration of 20 mM MOPS, 5 mM sodium acetate and 1 mM EDTA in 100 ml RNase free water. The running buffer was 1X FA gel. Electrophoresis was for 60 min at 70 V (Appendix G). The approximate intensities of the 28S and 18S rRNA bands, visible tRNA patches lower on the gel as well as the lack of high molecular weight DNA was used as an indication of good quality RNA suitable for cDNA synthesis.

3.1.4 cDNA synthesis and amplification of *mccA* and *mccB*

All the nucleic acid sequences of primers was used for PCR (Table 3.1) and sequencing (Tables 3.2 and 3.3) were from the literature. Oligo dT_{18mer} was used for cDNA synthesis from total RNA. Primers were designed to amplify of *GAPDH*. Primers were synthesized by the commercial companies Metabion, Tibmolbiol, and Inqaba Biotechnical Industries (Pty) Ltd. Upon receipt, lyophilized primers were reconstituted in master stocks to a final concentration of 100 µM in 18 Ω water (Millipore Q, RiOs Elix 10). Master stock solutions of the primers were stored at -20 °C. Working solutions of 10 µM were prepared and stored at -20 °C until needed.

3.1.4.1 Primer selection

Holtzinger *et al.*, 2001 and Buamgartner *et al.*, 2001, published primers for PCR and RT-PCR of the *mccA* and *mccB* genes. Specific exons were chosen from the published data, taking into account specific mutations that were suspected to be associated with the South African patient. Baumgartner *et al.*, 2001 listed the mutations and the ethnic group of the patients (Baumgartner *et al.*, 2001). Primer details for the first strand cDNA synthesis as well as the details of the positive control gene *GAPDH* are given in Table 3.1 and 3.4 respectively. The primer set MBMCCA1-19 amplifies a 2326 bp fragment which spans the entire open reading frame of the *mccA* gene (-51bp to +2275) and the MBMCCB1-17 primer set amplifies a 1923 bp fragment which spans (-99 to +1824), where +1 is the A of the initiation ATG codon.

TABLE 3.1: SEQUENCE OF PRIMERS USED FOR AMPLIFICATION OF *mccA* AND *mccB* MATURE mRNAs

Amplicon	Size (bp)	Primer	Length (bases)	T_m (°C)	Primer sequence (5' – 3')
mccA 1-19	2326	MB-MCCA1-19for	19	60,0	gACgCAgCTgCCTCTgTAC
		MB-MCCA1-19rev	19	62,0	gTgACCCAAATgCATgATTCT
mccB 1-17	1923	MB-MCCB1-17for	20	64,0	AggACCTgAgCTCAgCTTCC
		MB-MCCB1-17rev	21	62,0	ACTgTAACAgCCTCATgTTCCg

for , forward primer; rev , reverse primer (Baumgartner *et al.*, 2001)

TABLE 3.2: LIST OF THE SEQUENCING PRIMERS USED FOR SEQUENCING *mccA*

Gene	Primer Name	Primer Sequence (5' – 3')
*mccA*1-19	MB-MCCA1-19for	gACgCAgCTgCCTCTgTAC
	MCCA_S1_for	ATCTgCAATTAgAgACATggg
	MCCA_S2_for	AgAAgATTCCTTTgAgCCAgg
	MCCA_S3_for	gTTgTTgCTCAgTCggAAgg
	MCCA_S4_for	gATgggTCTTATAgCATgCAg
	MCCA_S5_rev	CATCCTggATggATAgCCTg
	MCCA_S6_rev	CAgATTTAATACCAggCgCTg
	MCCA_S7_rev	gTCTgCTCgAggAgTAgAgAg
	MCCA_S8_rev	gCTCCATCTTCATggCgATC
	MB-MCCA1-19rev	gTgACCCAAATgCATgATTCT

Baumgartner personal communication 2005)

TABLE 3.3: LIST OF THE SEQUENCING PRIMERS USED FOR SEQUENCING *mccB*

Gene	Primer Name	Primer Sequence (5' – 3')
*mccB*1-17	MB-MCCB1-17for	AggACCTgAgCTCAgCTTCC
	MCCB_S1_for	CCCAgTTTgCAggTTACCAg
	MCCB_S2_for	AACTggggAgAAgAAgTATCTgC
	MCCB_S3_for	TAACCCTCATCATTgggggC
	MCCB_S4_rev	CTggTAACCTgCAAACTggg
	MCCB_S5_rev	TACTTCTTCCCCAgTTgCCg
	MCCB_S6_rev	TAggAgCCCCCAATgATGAG
	MB-MCCB1-17rev	ACTgTAACAgCCTCATgTTCg

(Baumgartner personal communication 2005)

TABLE 3.4: SEQUENCE OF PRIMERS USED FOR AMPLIFICATION OF *GAPDH*

Amplicon	Size (bp)	Primer	Length (bases)	T_m (°C)	Primer sequence (5' – 3')
GAPDH	226	*GAPDH*-fwd	19	62,3	gAAggTgAAggTCggAgTC
		GAPDH-rev	20	58,4	gAAgATggTgATgggATTTC

GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; fwd, forward primer; rev, reverse primer

3.1.4.2 Reverse transcriptase polymerase chain reaction (RT-PCR)

The RT-PCR reaction can be done as a single reaction (one-step) or as a coupled reaction (two-step).

One-step RT-PCR

In a one-step RT-PCR reaction the reverse transcription and PCR is carried out in one reaction. This reduces the possibility of cross contamination of samples and pipetting errors. An advantage is that the total cDNA synthesized during RT is used as template for PCR and not only a fraction of the synthesized cDNA as in the case of two-step RT-PCR. Although the one-step reaction is quicker than the two-step RT-PCR reaction, the main drawback is that neither the reaction conditions for the reverse transcriptase nor the polymerase is optimal.

Two-step RT-PCR

Two-step RT-PCR, is performed at optimal conditions and PCR is also performed at optimal conditions. In this study the two-step RT-PCR was used. The 20 µl reverse transcription was performed as follows: a 13 µl reaction mixture was prepared that consisted of 5 µg total RNA, 1 mM dNTPs and 500 ng oligo dT_{18mer} primer. This mixture was denatured in an Eppendorf Master Cycler for 5 min at 65 °C and immediately snap-cooled on ice. To the mixture 4 µl cDNA synthesis buffer, containing Tris-acetate, potassium acetate and magnesium acetate was added as well as DTT, 40 units of RNase inhibitor and 15 units cloned AMV RT. The final concentration of the cDNA synthesis buffer components present in the 20 µl RT reaction mixture are 50mM Tris-acetate (pH8,4), 75 mM potassium acetate and 8 mM magnesium acetate was added as well as 50 µM DTT. The reaction mixture was carefully mixed and placed back into the Eppendorf Master Cycler for elongation for 60 min at 45 °C. The RT reaction was terminated by heating for 5 min at 85 °C.

After cDNA synthesis the amplification of the open reading frames of both *mccA* and *mccB* was performed using the gene specific primers listed in Table 3.1. The high fidelity DNA polymerase Phusion (cat no. F-530S, Finnezymes) was used for amplification. The Phusion polymerase has a error rate of 4,4 X 10^{-7} in 5 x HF buffer (cat no. F518, which contains 7,5 mM magnesium chloride

($MgCl_2$), resulting in a final concentration of 1,5 mM $MgCl_2$ for PCR and approximate 10 times less in 5 x GC buffer (cat no. F519), which contains 7,5 mM $MgCl_2$, which also resulting in a final concentration of 1,5 mM $MgCl_2$ for PCR. The rest of the components of the buffer is not revealed by manufacturer. Phusion polymerase produces blunt ends for further cloning procedures. The 50 µl PCR mixture consisted of: Phusion GC buffer containing a final concentration of 1,5 mM $MgCl_2$, 0,2 mM dNTPs, two gene specific primers at a final concentration of 0,5 µM each, 6 µl synthesized cDNA as template and 1 unit high fidelity Phusion DNA polymerase. Water was added to a final volume of 50 µl.

The first step for PCR was a denaturing cycle at 98 °C for 5 min followed by 35 cycles of denaturation at 98 °C for 10 sec, annealing at a temperature range from 58 to 60 °C, depending on the primers used, for 30 seconds and elongation at 72 °C for 90 seconds. A last elongation step at 72 °C for 10 min completed the amplification reaction. A cooling step of 4 °C ended the PCR programme. The amplified PCR product was analysed with agarose gel electrophoresis and when necessary, gel extraction was done as described in Section 3.1.5.

3.1.5 Gel extraction and sequencing of the *mccA* and *mccB* amplicons

The PCR amplicons were resolved by means of agarose gel electrophoresis. The relevant DNA fragments were purified with the Qiaquick® gel extraction Kit (Cat no. 28106 Qiagen, see Appendix M for step by step protocol). The gel extraction system is based on spin-column technology with selective binding properties of DNA to a membrane using salt and pH. The binding buffers provided the correct salt concentration and pH for adsorption of DNA to the membrane. The binding buffer contains a pH indicator to ensure that the pH of the solution is below or at 7.5, 3 M sodium acetate, pH 5, should be added if the solution changes color from yellow to violet. The sample was washed to remove salts, enzymes, unincorporated nucleotides, agarose, dyes, ethidium bromide from the DNA. An additional centrifugation step ensures the quantitatively removal of salts and ethanol containing buffer which may interfere with subsequent enzymatic reactions. The elution efficiency is strongly dependent on low salt concentration and pH values between pH7.0 and pH 8.5. DNA is eluted with buffer EB (10 mM Tris-Cl, pH8.5)(see Appendix M for a step by step laboratory protocol).

DNA sequencing was done at Inqaba Biotech (Pretoria, South Africa) and the Onderstepoort Veterinary Institute (Pretoria, South Africa). Sequencing data and chromatograms that were received from these facilities were very carefully analysed using the Chromas and Vector NTI (Scientific group, 2005) software.

3.2 RESULTS AND DISCUSSION

3.2.1 Isolation of total RNA

The isolation of total RNA was done with the RNeasy® total RNA isolation kit and with the Qiazol® lyses reagent (Section 3.1.2.). Cultured fibroblast cells of a MCC non-deficeint individual were used for the isolation of total RNA. The quality RNA was analysed on a non-denaturing 1 % agarose gel (Figure 3.4 a), and/or a 1,2 % formaldehyde denaturing gel (Figure 3.4 b). The yield and quality of the isolated total RNA was also determined by measuring the absorbance at A_{260} and A_{280} using a NanoDrop® spectrophotometer (Table 3.5).

TABLE 3.5: THE QUALITY AND QUANTITY OF THE ISOLATED TOTAL RNA FROM CULTURED FIBROBLASTS OF A MCC NON-DEFICIENT INDIVIDUALS USING ONE CONFLUENT 25 CM^2 FLASK FOR EACH ISOLATION

Starting material	Individual	Method	Concentration ng/µl	Total yield (µg) in 50 µl RNase free water	Ratio A_{260}/A_{280}
Fibroblast cell cultures ± 2 X 10^6 cells	CFC	RNeasy®	131,33	6,6	2,09
Fibroblast cell cultures ± 2 X 10^6 cells	CFC	Qiazol®	2521,33	126,1	2,00

The yield of the isolated total RNA with Qiazol® is much higher than with RNeasy®. No difference in the A_{260}/A_{280} ratios was observed.

Non-denaturing agarose gel electrophoresis was used to evaluate the integrity and overall quality of a total RNA preparation by inspection of the 28S and 18S rRNA bands. The secondary structure of RNA alters its migration pattern in native gels so that it will not migrate according to its true size, but two bands will, nevertheless be visible. Although agarose gel electrophoresis has drawbacks, but is sufficient to evaluate the integrity and overall quality of the isolated total RNA (Figure 3.4).

According to the RNeasy® manual a yield of 15 µg total RNA with an A_{260} / A_{280} of 2 should be expected when using 1 x 10^6 cells. The maximum capacity of the spin columns are 1 X 10^7 cells or 100 µg total RNA. Poor RNA yields can be expected when the columns are overloaded. To eliminate the possibility of overloading the columns, different amounts of cells were used for isolating total RNA. The cells from one 80% - 100% confluent 25 cm^2 flask (containing approximately 1 X 10^6 cells) was divided in half. One half (approximately 5 X 10^5 cells each) was used for total RNA extraction and the other was divided in two alliquots. Total RNA obtained from 5

X 10^5 cells and 1 X 10^6 cells yielded 3,3 µg and 2 µg respectively. These results corresponds to the yield obtained from 2 X 10^6 cells that yields in 6,6 µg total RNA isolated.

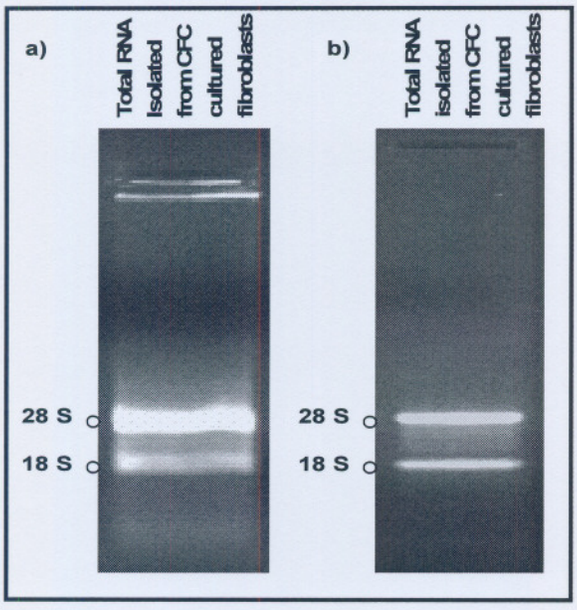


FIGURE 3.4: AGAROSE GEL ANALYSES OF ISOLATED RNA FROM CFC CULTURED FIBROBLAST CELLS. Lanes **a)** RNA isolated from CFC cultured fibroblasts with the Qiazol ® reagent was separated on a non-denaturing **b)** RNA isolated from CFC cultured fibroblasts using the Qiazol® reagent run on a 1,2 % formaldehyde denaturing gel.

A 75 cm^2 flask (approximately 6 X 10^6 cells) of cultured fibroblasts was also used for total RNA isolation using RNeasy®. The 75 cm^2 flask (± 6 X 10^6 cells) and the 25cm^2 flask (2 X 10^6 cells) both yielded 15 µg total RNA. Therefore it was assumed that the column was not overloaded in the case of the 25 cm^2 flasks but it has been in the 75 cm^2 flask. The expected yield for 6 X 10^6 cells is approximately 20 µg according to the amount of RNA isolated from an 80% - 100% confluent 25 cm^2 flask. Table 3.5 gives the average amount of total RNA isolated from an 80% - 100% confluent 25 cm^2 flask cultured fibroblast cells using the RNeasy® total RNA isolation kit. The yield was approximately 50% less than that claimed by the manufacturer (RNeasy® handbook, 2001, Qiagen).

Following the poor yield of total RNA isolated from cultured fibroblast cells using the RNeasy® total RNA isolation kit, a phenol-chloroform phase separation-based method, Qiazol® lyses reagent, was used. This was done in order to increase the quantity of total RNA isolated from the same amount of cells used isolating total RNA with the RNeasy® total RNA isolation kit. The use of the Qiazol® lyses reagent yielded in approximately 126,1 µg total RNA from 2 X 10^6 cells. This is 15 times the amount of total RNA isolated with RNeasy® using equal number of cells (Table 3.5). The purity was

spectrophotometrically determined using NanoDrop® (Section 3.1.3) and the total yield was calculated using equation 3.2.

A disadvantage of the phenol-chloroform based total RNA isolation method is the phenol-chloroform interference that may occur during RT-PCR amplification of transcripts. To overcome this problem, the isolated total RNA was cleaned up using the RNeasy® cleanup protocol (see Appendix I for a step by step protocol). A typical electrophoretogram of total RNA from cultured fibroblasts using Qiazol® lyses reagent is shown in Figure 3.4a and b. Two bands, 28S and 18S, are visible in both lanes. The 28S band appears to be twice as intense as the band representing the 18S ribosomal RNA. This indicates that good quality total RNA was isolated. The next step was to synthesize cDNA in order to amplify the open reading frames of the *mccA* and *mccB* genes.

3.2.2 Two-step RT-PCR

The two-step RT-PCR method (Section 3.1.4.2) was used. Oligo dT_{18mer} was used to synthesize cDNA while for PCR of *mccA* and *mccB*, specific primers were used for. The housekeeping gene, *GAPDH*, was included as a positive control. The amplicons were analysed on a 1 % agarose gel (Section 3.1.3.2). The positive control gave a single clear visible amplicon of 226 bp with no visible non-specific amplicons (Figure 3.5) present.

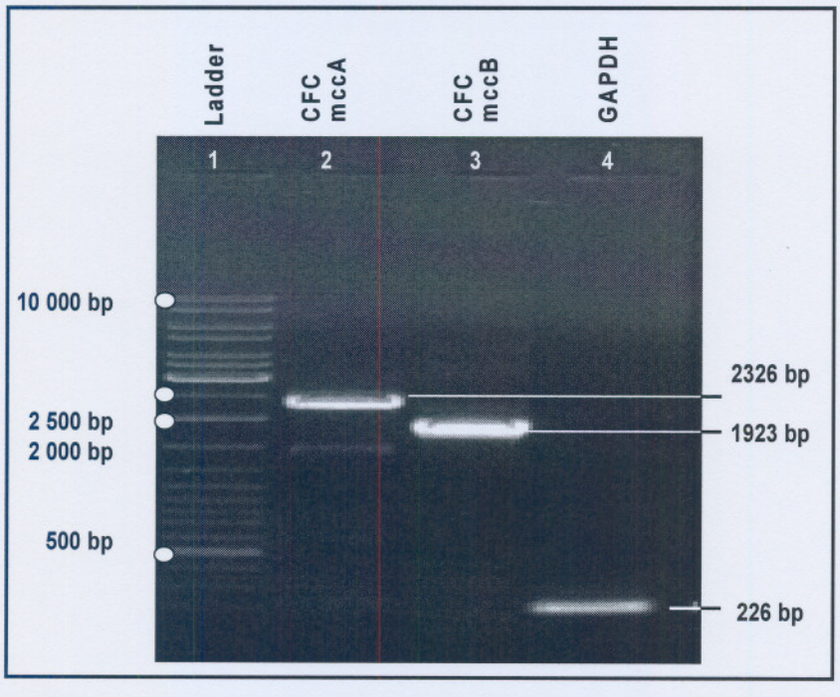


FIGURE 3.5: 1% AGAROSE GEL ANALYSES OF RT-PCR, AMPLIFIED CFC*mccA*, CFC*mccB* AND *GAPDH* GENES. Lanes: 1) DNA marker (O'GeneRuler, cat no SM 1173 Fermentas); 2) amplified *mccA*; 3) *mccB* and 4) amplified *GAPDH*

The amplification of the correct size *GAPDH* amplicon using gene specific primers indicated that cDNA synthesis from the oligo dT_{18mer} primer was successful. A few very faint non-specific amplicons were present in the amplicons of both *mccA* and *mccB*. In both *mccA* and *mccB* clear amplicons were present with the correct approximate sizes of 2326 bp and 1923 bp respectively. The presence of the non specific amplicons interferes with sequencing. Therefore, the specific amplicons were purified by gel extraction and sent for sequencing to confirm specificity.

3.2.3 Sequence Analyses

Each primer covers approximately 400 bases before overlapping with the next primer. Sequencing primers for the coding and non-coding strands are included. The sequence data and chromatograms of the respective genes were carefully analysed and aligned with related published sequences. The convention numbering for the nucleotides are given where the A of the ATG (initiation codon of the ORF) is 1. The last nucleotide coding for the last amino acid in the ORF before the termination codon (TAA) is the last nucleic acid of the ORF. Therefore bases from the ATG to the 5' UTR is (-X bp) and from the TAA to the 3' UTR +X bp.

3.2.3.1 Sequences of CFC*mccA*

The alignment of CFC*mccA* with AF310339 and AF310972 showed two sequence differences. One was observed in the ORF and one in the 3' untranslated region (UTR) (Figure 3.6.). Both nucleotide differences in CFC*mccA* corresponded to published sequences. However, none of the published sequences contains both these differences. Figures 3.7 and 3.8 confirm the variations in the CFC*mccA* amplicon. The nucleotide change of 1391C→A in CFC*mccA* (referred to AF310972) is a known non-synonymous SNP resulting in a change in the amino acid sequence of the alpha subunit of MCC, i.e. H464P (Gallardo *et al.*, 2001, GenBank, 2005).

The other sequence variation (2175+131 bp) that was observed in the amplified CFC*mccA* is present in the 3'UTR regions of the *mccA* ORF and did not affect the sequence of the translated protein. Unfortunately, only the coding strand sequence was obtained for this region of the *mccA* ORF. The primer to sequence the reverse strand was too close to this position to confirm the nucleic acid variation that was observed in the forward sequence. No other sequence differences were detected in the sequence chromatograms and with the alignment analyes.

The entire deduced amino acid sequence AF310339 *mccA* indicating the variation of H464P CFC*mccA* is depicted in Appendix A.

FIGURE 3.6: SEQUENCE ALIGNMENT OF CFC*mccA* WITH GENBANK SEQUENCES AF310339 AND AF310972. Highlighted ATG, ORF of *mccA*1-19 begin and TAA, termination of translation or stop. Highlighted ATG, ORF of CFC*mccB* initiation and TAA , termination of translation or stop Green: UTR not amplified; Purple: Initiation codon (ATG) and the termination codon (TAA); Grey: Primer sequences; Red: Position 1391 A →C.

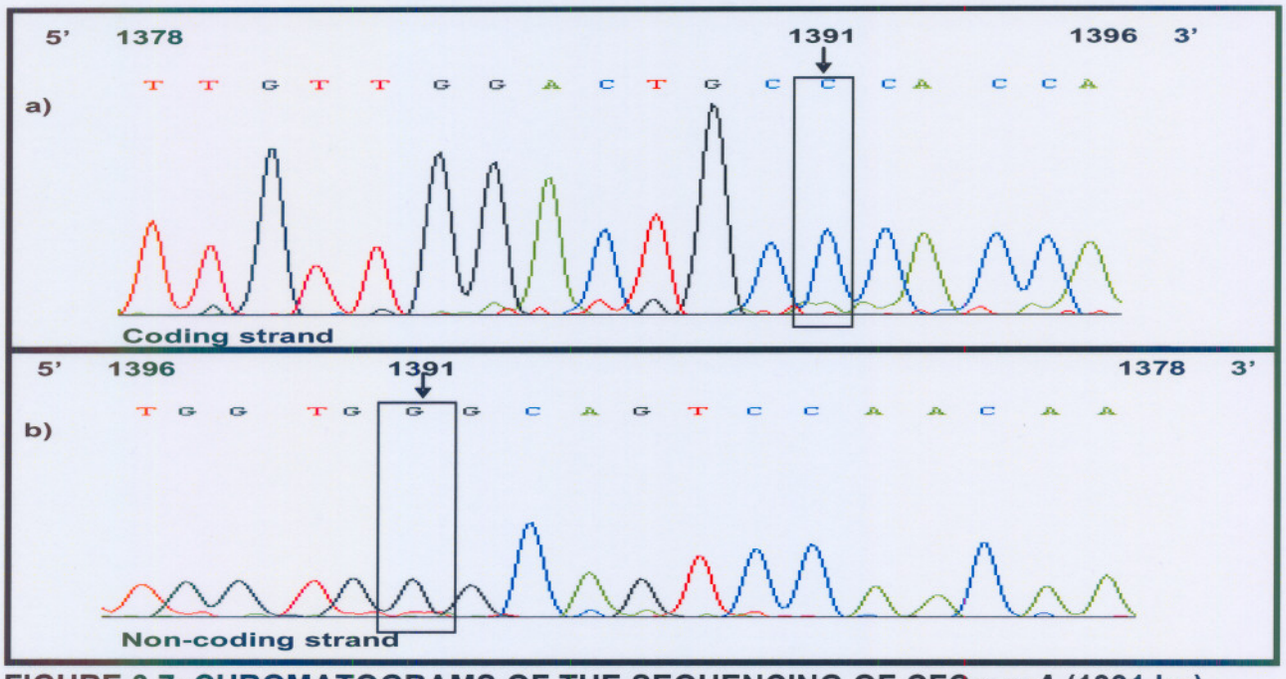


FIGURE 3.7: CHROMATOGRAMS OF THE SEQUENCING OF CFC*mccA* (1391 bp)
Highlighting the cytosine of the a) coding strand and guanine of the b) non-coding strand in position 1391 of the CFC*mccA* ORF.

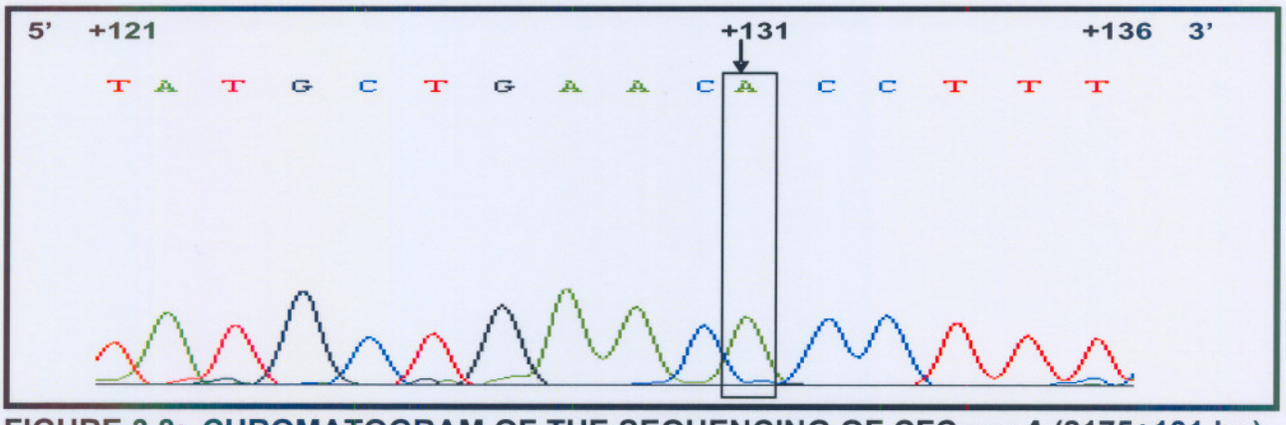


FIGURE 3.8: CHROMATOGRAM OF THE SEQUENCING OF CFC*mccA* (2175+131 bp)
Highlighting the adenine in position 2175+131 of CFC*mccA* ORF.

3.2.3.2 Sequences of CFC*mccB*

The sequence of CFC*mcc*B was analysed and aligned with the published sequence AF310971 (Figure 3.9). There was only one nucleotide variation (1368G→A) observed. To confirm this mutation, both strands of CFC*mcc*B were sequenced. Chromatograms of the forward strand as well as the sequence of the reverse strand are given in Figure 3.10. This Figure clearly showed that the nucleic acid in position 1368 is an adenine and not a guanine. This nucleotide change 1368G→A is not yet described in the literature but has recently been listed in the GenBank SNP database (Baumgartner *et al.*, 2001; Gallardo *et al.*, 2001; Holzinger *et al.*, 2001, Dantas *et al.*,

2005, GenBank 2006). The mutation 1368G→A, A456A is in the wobble position of this Ala codon and does not result in an amino acid change (Figure 3.11). Therefore it is considered as a synonymous SNP. The entire deduced amino acid sequence of AF310791*mcc*B and/or CFC*mcc*B is given in Appendix B.

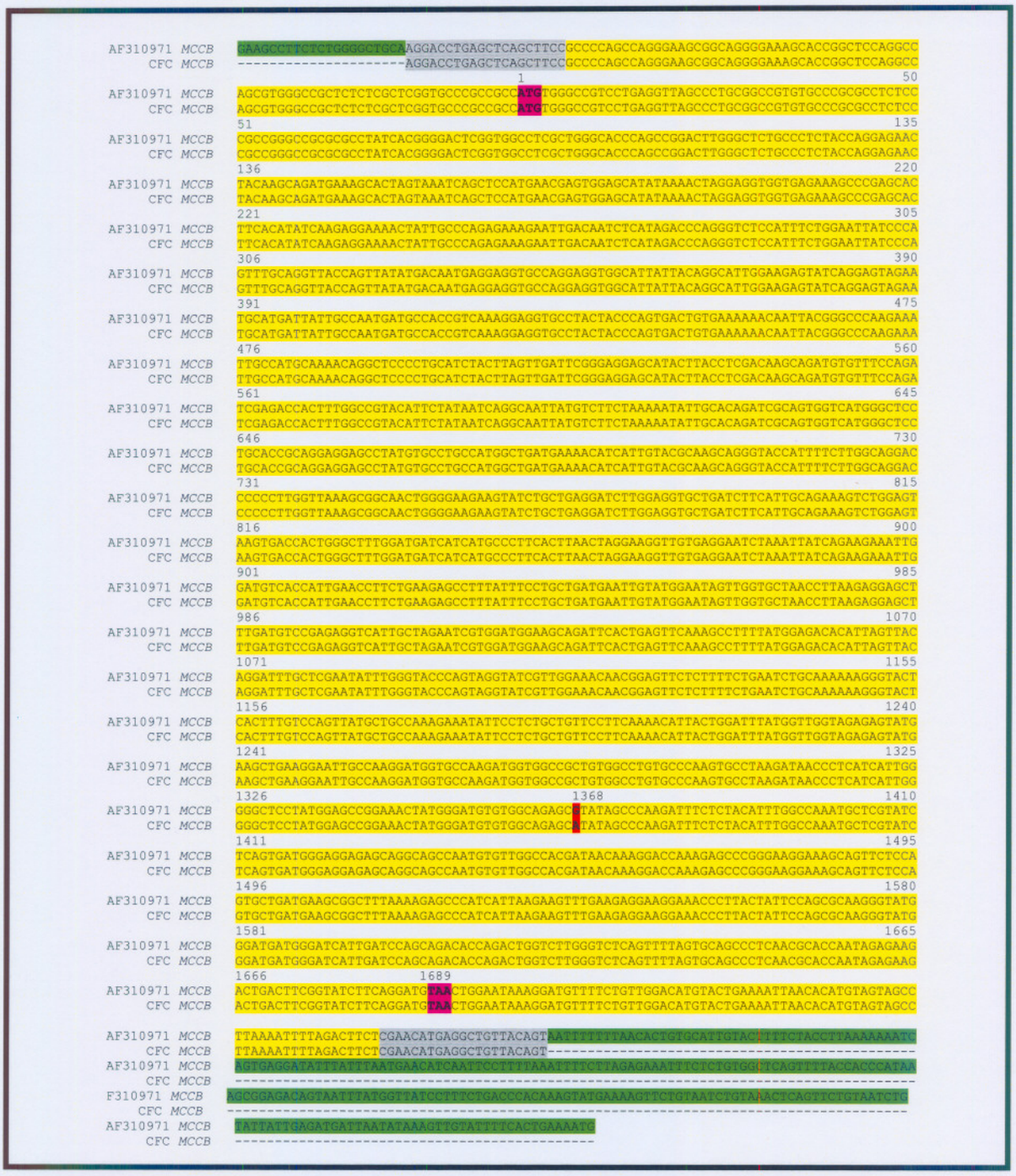


FIGURE 3.9: SEQUENCE ALIGNMENT OF CFC*mccB* WITH GENBANK SEQUENCE AF310971. Highlighted ATG, ORF of CFC*mccB* initiation and TAA , termination of translation or stop. Green: UTR not amplified; Purple: Initiation codon (ATG) and the termination codon (TAA); Grey: Primer sequences; Red: Position 1368 G→A.

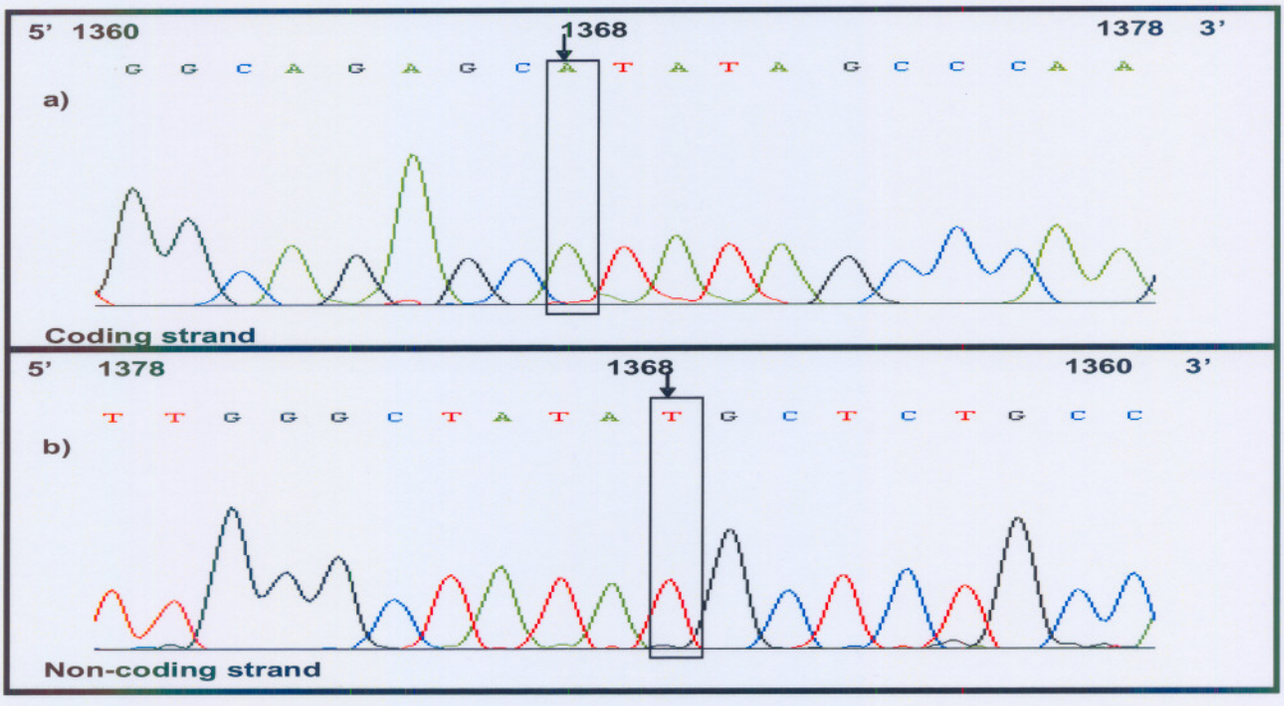


FIGURE 3.10: CHROMATOGRAMS OF THE SEQUENCING OF CFC*mccB* (1368 bp)
Highlighting the adenine of the a) coding strand and guanine of the b) non-coding strand in position 1368 of the CFC*mccB* ORF.

```
1       436                                                  456                STOP
Met....LysIleThrLeuIleIleGlyGlySerTyrGlyAlaGlyAsnTyrGlyMetCysGlyArgAlaTyrSerPro........***
ATG....AAGATAACCCTCATCATTGGGGGCTCCTATGGAGCCGGAAACTATGGGATGTGTGGCAGAGCGTATAGCCCA........TAA
a)

1       436                                                  456                STOP
Met....LysIleThrLeuIleIleGlyGlySerTyrGlyAlaGlyAsnTyrGlyMetCysGlyArgAlaTyrSerPro........***
ATG....AAGATAACCCTCATCATTGGGGGCTCCTATGGAGCCGGAAACTATGGGATGTGTGGCAGAGCATATAGCCCA........TAA
b)
```

FIGURE 3.11 ALIGNMENT OF THE DEDUCED AMINO ACID SEQUENCE OF a) AF310791 *mccB* AND b) CFC*mccB*. Ala 456 is in red bold and the changed nucleotide is highlighted

3.3 SUMMARY

Good quality total RNA was isolated from culutred fibroblast cells using the Qiazol® reagent. To eliminate the interference of phenol and chloroform during RT-PCR the total RNA preparation was cleaned with RNeasy® columns using the cleanup protocol. Native agarose and denaturing formaldehyde agarose gel electrophoretic analyses showed sharp 28S and 18S rRNA bands (Figure 3.4). The 28S rRNA band appears approximately twice as intense as the 18S rRNA band. This 2:1 ratio (28S:18S) is a good indication that the RNA is intact. Partially degraded RNA will smear, it will lack the sharp rRNA bands, or it will not have a 2:1 ratio. Completely degraded RNA will appear as a very low molecular weight smear (Applied biosystems, 2005). Spectrophotometric

quantification of the isolated total RNA correlated with the gel analyses. The isolated total RNA was used for first strand cDNA synthesis. The amplification of both transcripts i.e. (CFC*mccA* and CFC*mccB*) of the control individual, CFC, was successful.

Due to the presence of very faint non-specific amplicons (Figure 3.5), gel extraction was done to purify both the *mccA* and *mccB* amplicons from the non-specific amplicons. This excluded the possibility that mixed templates would render the sequence results null and void.

Two nucleotide differences were observed between the CFC*mccA* sequence and that of two of the published *mccA* sequences. Only one of the changes was in the ORF, the other was in the 3' UTR of the amplified CFC*mccA* (Figures 3.6, 3.7 and 3.8). The nucleic acid change 1391A→C of CFC*mccA* resulted in the H464P amino acid change. This missense mutation 1391A→C, His464Pro is one of two frequent polymorphisms documented for *mccA*. The other is the synonymous point mutation 369T→C; L132L (Gallardo *et al.*, 2001).

The sequence chromatograms of CFC*mccB* was carefully analysed and one nucleotide difference was observed in the alignment of CFC*mccB* with AF310791 (Figures 3.9, 3.10 and 3.11). The nucleic acid change 1368G→A of CFC*mccB* does not change the amino acid sequence and is therefore considered as a synonymous SNP. This SNP was recently listed in the GenBank SNP database (17 November 2006).

The results confirmed that the experimental procedures for the RT-PCR of *mccA* and *mccB* and their molecular characterisation through sequencing were implemented succesfully. The next step was to use this technology to characterize the mature mRNA transcripts *mccA* and *mccB* of a South African (HGS) with mild MCC deficiency. This is described in Chapter 5.

Chapter Four

Molecular characterisation of selected *mccA* and *mccB* exons

Since the MCC genes (*mccA* and *mccB*) of patients throughout the developed world are being well characterized and documented it is important for the RSA to have the same level expertise to complement its biochemical MS/MS based programmes. The first part of a two-pronged approach applied to study MCC deficiency in SA patients, the molecular characterisation of the mature mRNA transcripts of the two MCC genes (*mccA* and *mccB*) (Chapter 3). In this chapter the focus will be to characterise the MCC on the genomic level to further confirm the results obtained from mature mRNA transcripts (Chapter 2). Four of the 36 exons were chosen to start the implementation of this approach. These exons (*mccA*8, *mccA*11, *mccB*5, *mccB*6) were selected since the identified mutations in these exons are mostly carried by Caucasian individuals with MCC deficiency. The South African patient with MCC deficiency is Caucasian. It is therefore likely that he has mutations in these exons. The intron between exon 5 and 6 of the beta subunit *mccB*, was also selected for amplification. This intron 5, carries a mutation (In5ac-1G→A) that is also associated with a Caucasian MCC deficient individual (Baumgartner *et al.*, 2001). Since the region spanning *mccB*5 and *mccB*6 together with intron 5 is less than 3000 bp, the region spanning exons 5 intron 5 and exon 6 was amplified. Thus three regions of interest could be analysed from one amplicon. Mutations described in association with these exons are listed in Tables 2.2. and 2.3.

The basic experimental design for the molecular characterisation of MCC genes is as follows. The Flexigene® gDNA isolation kit is mostly used to isolate gDNA. Amplification of exons is done using primers in the flanking intronic regions of the exon (Holzinger *et al.*, 2001; Baumgartner personal communication). A variety of DNA polymerases are used. The amplicons are directly sequenced. Thereafter mutation analyses are performed using appropriate computer software.

The final aim of this part of this study was to identify the mutation(s) in a South African patient having MCC deficiency. However, in this chapter, gDNA from MCC non-deficient individual CFC was used to implement the basic procedures. Cultured fibroblasts from CFC were used to isolate gDNA to serve as template for the optimisation of amplification conditions. A flow diagram (Figure 4.1) illustrates an outline of this Chapter.

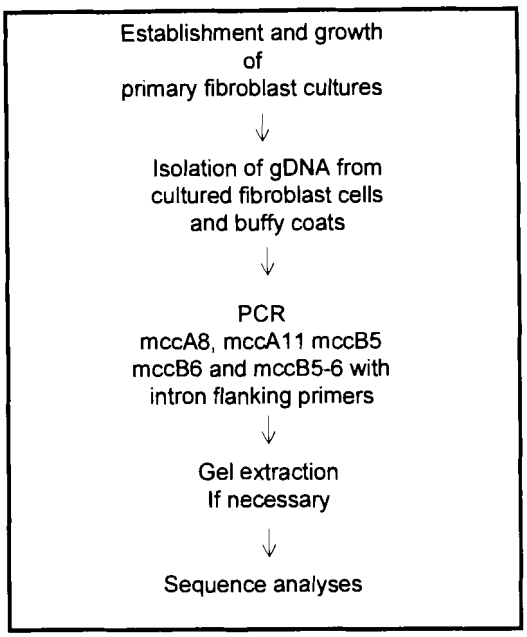


FIGURE 4.1: Experimental approach for the molecular characterisation of selected *mccA* and *mccB* exons (*mccA*8, *mccA*11, *mccB*5, *mccB*6 and *mccB*5-6).

4.1 MATERIALS AND METHODS

4.1.1 Isolation of gDNA

gDNA was isolated from cultured fibroblast cells and buffy coat using the Flexigene® genomic DNA isolation kit (cat no. 51206, Qiagen). The principle of this method will be outlined here whereas Appendix H gives the step by step laboratory protocol for gDNA isolation (Flexigene® DNA handbook, Qiagen 2003).

Cultured fibroblast cells and/or buffy coat were used as starting material to isolate gDNA isolation. After the addition of the cell lyses buffer to cells denaturing buffer was added. It contains choatropic salts and proteases to denature proteins. After several extractions RNA was still present in the purified gDNA preparations. Thus cell lyses was often not complete. Therefore, the gDNA isolation procedure was modified to include an extra centrifugation step after cell lyses to remove all unlysed cells. Incubation of the lysed cells was followed by an additional centrifugation[1] step to ensure that unlysed cells were discarded and did not interfere with the isolation process (see centrifugation[1] step Figure 4.2). An alcohol precipitation was done on the supernatant to concentrate, desalt and recover nucleic acids. The precipitation of gDNA was mediated by the presence of high salt concentrations (final concentrations of 0,3 M sodium acetate or 2,0 – 2,5 M ammonium acetate). The DNA precipitate was collected at the bottom of the tube by centrifugation. The supernatant was discarded. The DNA was then washed with 70 %ethanol in order to remove remaining remianing salts and traces of protein. gDNA was then left to dry. It was then

resuspended in a rehydration buffer (10 mM Tris-Cl, pH8,5) (Flexigene® DNA handbook Qiagen February 2003).

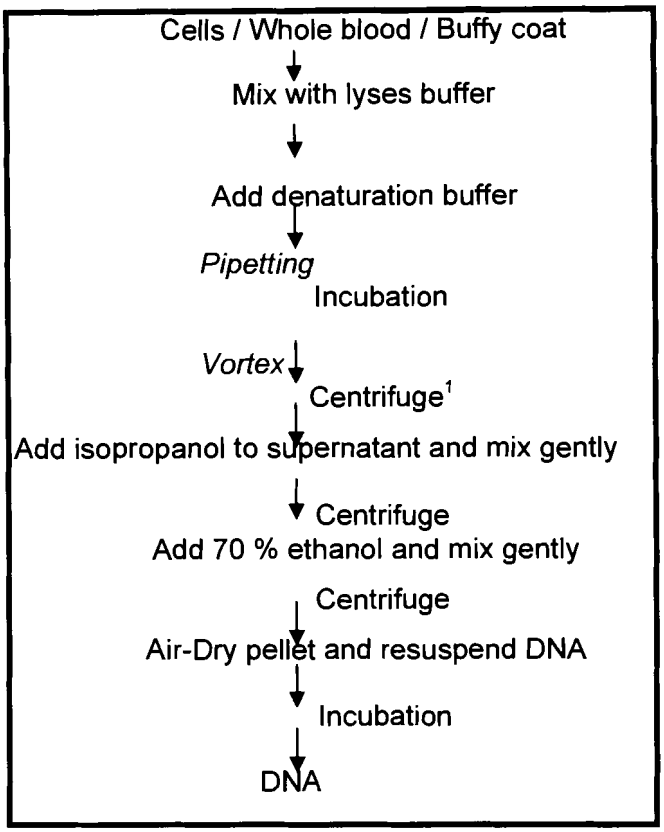


Figure 4.2: Flow diagram of the general DNA isolation steps
(Flexigene® handbook February, 2003).

A 80 -100 % confluent 25 cm^2 flask of cultured fibroblasts contain approximately 1-3 x 10^6 cells was used. Since fibroblasts are not listed in the Flexigene® handbook, therefore the yield and purity that is expected from 1 X 10^6 HeLa cells was taken as a guideline of how much gDNA to expect from an equal number of fibroblasts. The expected yield of gDNA from 1 X 10^6 HeLa cells is 25 – 35 µg (Flexigene® Handbook 2003). Buffy coats from healthy adult individuals were also used to isolated gDNA. Blood samples were obtained from three healthy adult volunteers (LZ, EVD and CVH). A qualified nurse at a commercial clinical pathology laboratory (AMPATH Potchefstroom, NWU Building F3) collected the blood. Approximately 3 ml blood was collected in 4 ml ethylenediamine tetra-acetic acid (EDTA Cat no. 368861, Transgen) or heparin (Cat no. 367526, Transgen) coated tubes. Directly after the blood was collected it was thorougly mixed to avoid coagulation. It was placed on ice (±4 °C) to slow down all enzymatic and physiological processes. Blood was then spotted on each circle of 15 mm diameter on Guthrie cards. To saturate one circle, 40 µl blood was applied to a circle. For each individual three cards were prepared. The Guthrie

cards were then left in a cool dry place to dry. The remaining blood was immediately used for buffy coat isolation to isolate gDNA.

4.1.2 Preparation of buffy coats

Histopauqe® (cat no. 1077, Sigma- Aldrich) was used to prepare buffy coats. It is a solution of polysucrose and sodium diatrizoate, adjusted to a density of 1,077 ± 0.001 g/ml. In this medium the different blood components separate from each other in three phases (upper, interphase and lower phase) upon centrifugation. The upper phase consists of plasma and the lower phase of the red blood cells. The interphase is the phase in which the white blood cells are collected. The interphase can be subdivided into the Histopaque® phase and the protein phase, between the upper plasma and the middle Histopaque® phase (Information leaflet for Histopaque, SIGMA-ALDRICH, 2005). This is collectively called the buffy coat. Approximately 1 ml buffy coat is recovered from 2 ml of whole blood. The buffy coat was divided into 1 ml aliquots. One was immediately used to prepare gDNA and the others were stored at -80 °C for later use. Before the buffy coats were used, the cells were washed with 1X PBS and pelleted.

4.1.3 Quantification and characterisation of gDNA

For a double stranded DNA (dsDNA) preparation, one OD_{260} corresponds to 50 $ng.\mu l^{-1}$. Spectrophotometric analysis was done on a NanoDrop® instrument (NanoDrop® ND1000). The purity of DNA is confirmed by the A_{260}/A_{280} ratio. When dsDNA preparations are pure, the A_{260}/A_{280} ratio is 1.8. The concentration of isolated gDNA can be calculated using equation 4.1. The total yield obtained from the isolation is calculated using equation 3.2 (Section 3.1.3.1).

EQUATION 4.1: CALCULATION OF THE DNA CONCENTRATION FORM THE ABSORBANCE AT 260 NM

$$[\text{double-stranded DNA}] = A_{260} \times (50\ ng.\mu l^{-1} \times \text{dilution factor})$$

[DNA], DNA concentration; A_{260}, absorbance of samples at 260 nm (Adapted from Sambrook and Russell 2003)

After the spectrophotometric quantification of the isolated gDNA, the gDNA was electrophoretically analysed using a 1 % TAE agarose gel (Section 3.1.3.2.)

4.1.4 Amplification of exons *mccA*8, *mccA*11, *mccB*5, *mccB*6 and genomic region *mccB*5-6

TaKaRa Ex Taq polymerase (cat no. RR001A, TaKaRa) was used or the amplification of *mccA*8, *mccA*11, *mccB*5, *mccB*6 and *mccB*5-6 rather than Phusion DNA polymerase which is more expensive.

The DNA polymerase TaKaRa Ex Taq (cat no. RR001A, TaKaRa) produces amplicons with 3' A overhangs for A/T cloning procedures. The 50 µl PCR mixture consisted of 2 mM $MgCl_2$; 0,2 mM dNTPs; two gene (exon) specific primers (Table 4.1) at a final concentration of 0,5 µM each; approximately 400 ng gDNA as template, 1,25 units TaKaRa Ex Taq DNA polymerase. The first step for PCR was a denaturation step at 98 °C for 5 min followed by 30 cycles comprised of denaturation at 98 °C for 10 sec, annealing at a temperature between 55 – 60 °C depending on the specific primers that was used for 30 seconds and elongation at 72 °C for 60 seconds. A last elongation step at 72 °C for 10 min completed the amplification reaction. A cooling step of 4 °C ended the PCR programme (Table 4.2). In those cases where $MgCl_2$ concentration series were needed, the polymerase TaKaRa Taq with an $MgCl_2$ free buffer - (cat no. R001AM TaKaRa) was used.

Table 4.1: Sequence of primers utilized for amplification of specific exons

Amplicon	Size (bp)	Primer	Length (bases)	T_m (°C)	Primer sequence (5' – 3')
mccA exon 8	245	[a]MCCA8for	20	56,3	CCTAACAgTTTAgCTTAACg
		[a]MCCA8rev	21	56.7	CAgAgTAAgATTCACATTACg
mccA exon 11	281	[a]MCCA11for	23	62,8	gTAgCCgCTATACggTAgATTTC
		[a]MCCA11rev	26	58,9	gTACTAAAACTTAAAAAgAgTgAgAC
mccB exon 5	425	[b]MB-*MCCB*5for	22	62,0	gACTgCTgTCTgCTAATggATg
		[b]MB-*MCCB*5rev	20	62,0	CCCCAgAgTTTTCAgCAAAg
mccB exon 6	383	[b]MB-*MCCB*6for	22	62,0	TAACAgTTTAgAAAgACAgggC
		[a]*MCCB*6rev	24	64,6	AgCCATCCCAgAgTACCTAATTCg
mccB exon 5 - 6	2123	[b]MB-*MCCB*5for	23	59,2	gACTgCTgTCTgCTAATggATg
		[a]*MCCB*6rev	24	64,6	AgCCATCCCAgAgTACCTAATTCg

MCCA, α-subunit of 3-methylcrotonyl-CoA carboxylase; *MCCB*, β-subunit of 3-methylcrotonyl-CoA carboxylase; for, forward and rev, reverse ([b] Baumgartner personal communication; [a] Holzinger *et al.*, 2001).

Following the amplification of the exons, they were electrophoretically analysed using a 1 % TAE agarose gel (Section 3.1.3.2.). Sequences obtained from direct sequencing were critically analysed using *Chromas* and *Vector NTI*.

Table 4.2: The conditions for PCR amplification of exons *mccA*8, *mccA*11, *mccB*5, *mccB*6 and *mccB*5-6

PCR step	Number of cycles	Action	Temperature (°C)	Duration (min : sec)
1	1	Denaturation	98	1:00
2	30	Denaturation Annealing Elongation	98 X 72	0:10 0:30 1:00
3	1	Elongation	72	10:00
4	1	Cooling	4	HOLD

X, annealing temperature specific for every exon, 55,2 °C for mcc*A*8; 57,5 °C for mcc*A*11; 57,8 °C for mcc*B*5 and 59,1°C for both mcc*B*6 and mcc*B*5-6.

4.2 RESULTS AND DISCUSSION

4.2.1 Isolation of gDNA from cultured fibroblasts and buffy coats

Genomic DNA was isolated from cultured fibroblast cells as well as from buffy coat obtained from several individuals using a modified Flexigene® (Qiagen) procedure (Section 4.1.1). When gDNA was prepared following the recommended procedure, the yield and the A_{260} / A_{280} ratio were variable. Ribosomal RNA (rRNA) was often also present when samples were analysed on 1% TAE agarose gels. This could be due to the lack of complete initial lyses of the cells. The cells then lysed later during the denaturing step of the procedure at which point RNase and protease would not be able to digest the RNA and proteins that is then released. An additional centrifugation was introduced to remove the unlysed cells. This yielded gDNA preparations that were pure while the yields were constant. With the addition of the additional centrifugation step directly after the lyses, the yield increased and the rRNA bands disappeared. A few examples of gDNA prepared from cultured fibroblast cells and buffy coat using the modified procedure are shown in Figure 4.3. These samples were analysed using a 1 % agarose gel electrophoresis. Spectrophotometric analyses were done using a Nanodrop® instrument. Results are shown in Table 4.3.

The amount of gDNA isolated from a one 25 cm^2 flask of cultured fibroblast cells was always approximately 40 µg. All the gDNA preparations had a A_{260}/A_{280} ratio of approximately 1,8, which is an indication of good quality gDNA. Although gDNA was isolated from three different fibroblast cultures and from buffy coat of three healthy individuals, only the gDNA isolated from the CFC cultured fibroblast cells was used for optimisation of the basic procedures. gDNA isolated from the buffy coats of LZ and CVH was subsequently used to verify the robustness of the optimized conditions.

TABLE 4.3: The quality and quantity of the DNA preparations from culture fibroblast cells and buffy coat

Starting material	Individual	Concentration ng/µl	Total yield (µg) in 200 µl F3 rehydration buffer (10 mM Tris-Cl, pH 8,5)	Ratio 260 / 280
Cultured fibroblast cells	CFC	198,48	39,7	1,76
Cultured fibroblast cells	ZL	188,00	37,6	1,87
Cultured fibroblast cells	MS	196,81	39,4	1,76
Buffy coat	LZ	188,71	37,7	1,88
Buffy coat	CVH	134,97	27,0	1,86
Buffy coat	EVD	175,11	35,0	1,93

In the case of gDNA isolated from buffy coat the modified extraction method gave good quality gDNA but the yield was only 30 % (± 30 µg) of the expected 110-260 µg for 1 ml buffy coat.

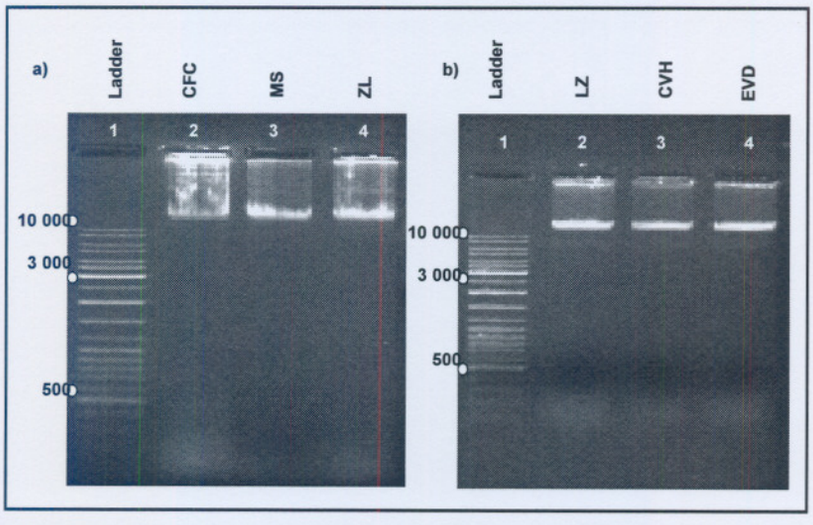


FIGURE 4.3: 1 % Agarose gel analyses of DNA isolated from (a) cultured fibroblas and (b) buffy coat with the Flexigene® DNA isolation kit. 5 µl of each 200 µl DNA sample was load on the gel. Lanes a1 and b1) O'GeneRuler (cat no. SM 1173, Fermentas); a2) CFC; a3) MS and a4) ZL; b2) LZ; b3) CVH and b4) EVD

4.2.2 Amplification of exons *mccA*8, *mccA*11, *mccB*5, *mccB*6 and genomic region *mccB*5-6

To identify mutations associated with the MCC deficiency on the genomic level associated with MCC deficiency five regions were selected to be amplified. It were exons *mccA*8 (245 bp), *mccA*11 (281 bp), *mccB*5 (425 bp), *mccB*6 (383 bp) and one region including exon5 intron 5 and exon6 (*mccB*5-6, 2123 bp), of the beta subunit were also selected. Published an unpublished primer sequences were used for the amplification of these five regions (Holzinger *et al.*, 2001; Baumgartner personal communication). Temperature and the Mg^{2+} concentration are important parameters in the optimisation of PCR conditions. This is due to their influence on the specificity of

primer annealing. The melting temperatures of the primers provided by the company that synthesized the primers were used as the starting point to optimize the reaction conditions for the amplification of each fragment. As initial annealing temperature of the primer set, a temperature 4 ºC below the average melting temperature of the two primers was used.

The initial reaction conditions for amplification of exons *mccA*11 and the *mccB*5-6 genomic region were not optimal. A first step to optimise reaction conditions was to perform PCR using a temperature gradient. A temperature gradient was prepared ranging from 55,8 ºC to 60,8 ºC (which is the average Tm of the two primers). The increase in temperature was approximately 2 ºC each (Figure 4.4). A faint amplicon was detected approximately 300 bp at the temperature of 57,5 ºC.

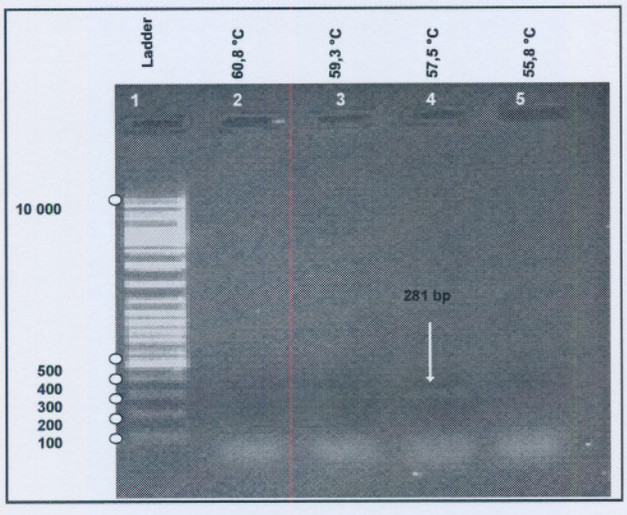


FIGURE 4.4: 1% Agarose gel analyses of amplicons obtained using a temperature gradient for the amplification of *mccA*11. Exon 11 of the alpha subunit of MCC was amplified at different temperatures. 5 µl of each 50 µl sample were loaded on the gel. Lanes 1) DNA marker O'GeneRuler (cat no. SM 1173, Fermentas); amplicons of *mccA*11 from PCR with an annealing temperature of 2) 60,8 ºC, 3) 59,3 ºC, 4) 57,5 ºC and 5) 54,8 ºC.

The expected size of the amplified *mccA*11 is 281 bp. It was assumed that this is the correct amplified fragment. However a band of smaller than 100 bp was also visible. This is due to the presence of unincorporated primers, since conditions were not optimal for PCR. Next, a set of PCRs were done to optimize the Mg^{2+} concentrations for the amplification of *mccA*11. The $MgCl_2$ concentration series used ranged from 0 mM to 5 mM $MgCl_2$ with incremental increases of 0,5 mM. The PCR result to determine the optimal $MgCl_2$ concentration for the amplification of *mccA*11 is depicted in Figure 4.5. Amplicons of approximately 300 bp were visible at $MgCl_2$ of 3,0 mM $MgCl_2$ and higher. Since amplification of *mccA*11 was achieved at 3,0 mM to 5,0mM $MgCl_2$ with no change in the intensity of the amplicons, the $MgCl_2$ concentration of 3,0 mM $MgCl_2$ was used in further experiments.

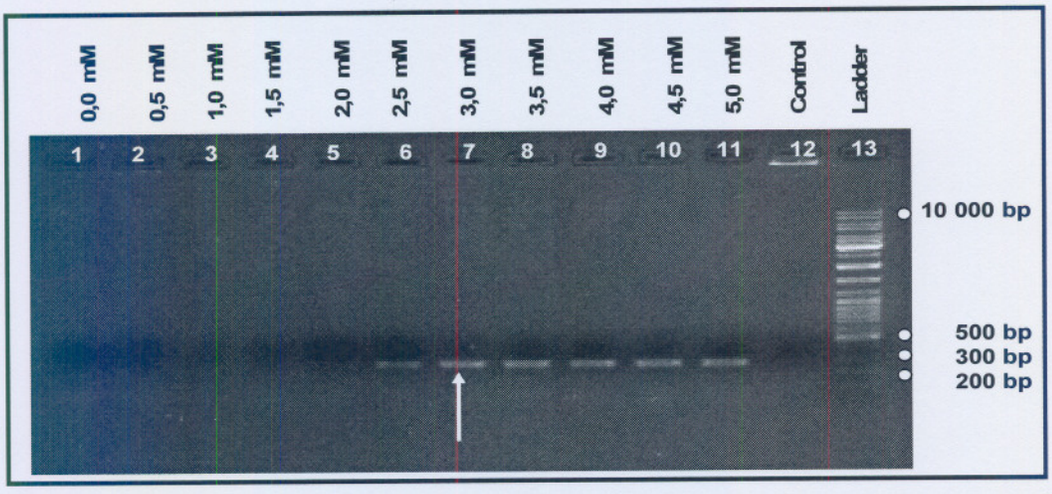


FIGURE 4.5: 1 % Agarose gel analyses of a $MgCl_2$ concentration series for the amplification of *mccA*11 of gDNA of CFC cultured fibroblasts at an annealing temperature 57,5 °C. Each lane represents PCR performed at a different concentration $MgCl_2$. 5 µl of each 50µl sample wasloaded. The concentration $MgCl_2$ was as follows: Lanes 1) 0,0 mM $MgCl_2$, 2) 0,5 mM $MgCl_2$, 3) 1,0 mM $MgCl_2$, 4) 1,5 mM, $MgCl_2$ 5) 2,0 mM, $MgCl_2$ 6) 2,5 mM, $MgCl_2$ 7) 3,0 mM, $MgCl_2$ 8) 3,5 mM, $MgCl_2$ 9) 4,0 mM, $MgCl_2$ 10) 4,5 mM, $MgCl_2$ 11) 5,0 mM, $MgCl_2$ 12) Control without primers and 13) DNA marker O'GeneRuler (cat no. SM 1173, Fermentas),

The optimisation of PCR conditions for the amplification of *mccB*5-6 was done by preparing a temperature gradient ranging from 53,2 °C to 63, 6 °C with incremental increases of approximately 2 °C. The temperature gradient was done at a $MgCl_2$ concentration of 1,5 mM in each 50 µl. A clear band of approximate 2000 bp was visible at an annealing temperature of 59,1 °C. These optimisations are not shown. The PCR of exons *mccA*8, *mccB*5 and *mccB*6 amplified satisfactory using final concentrations of 0,5 µM of each primer, 1,5 mM $MgCl_2$ in a 50 µl PCR mixture with annealing temperatures listed in Table4.5.

TABLE 4.4: Summary of amplification requirements of each amplicon

Amplicon	Size (bp)	Final concentration $MgCl_2$ (mM)	Annealing temperature (°C)
*mccA*8	245	1,5	52,3
*mccA*11	281	3,0	57,5
*mccB*5	425	1,5	57,8
*mccB*6	383	1,5	59,1
*mccB*5-6	2179	1,5	59,1

The amplicons were analysed on 1% agarose gels to verify the expected sizes. The size of the amplicon was determined by sizing the fragments against a DNA molecular weight marker (O'GeneRuler, cat no. SM 1173 Fermentas). The yield was high and non-specific amplicons were absent (Figure 4.6). Having optimized the PCR condition for the four amplicons as well as genomic

region *mccB*5-6 using cultured fibroblast cells of CFC, the next step was to determine how robust the conditions were for other individuals.

Genomic DNA was prepared form buffy coats of LZ and CVH. All four exons (*mccA*8, *mccA*11, *mccB*5, *mccB*6) and *mccB*5-6 amplified satisfactory from gDNA isolated from buffy coat of two individuals (LZ and CVH). No non specific amplification was present and the approximate size of the amplicons expected was obtained.

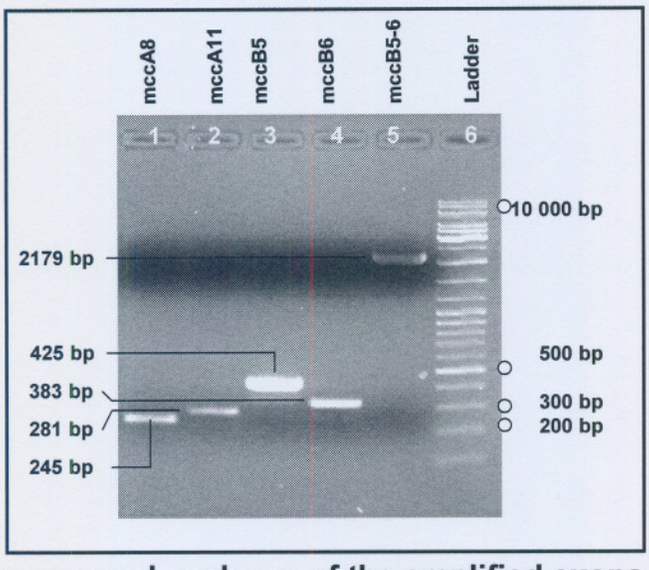


FIGURE 4.6: 1% Agarose gel analyses of the amplified exons from CFC cultured fibroblasts gDNA. Each lane consists of 5 µl of each 50 µl sample. Lanes 1); *mccA*8; 2) *mccA* 11; 3) *mccB*5; 4) *mccB*6; 5) *mccB*5-6 and 6) DNA marker O'GeneRuler (cat no. SM 1173,Fermentas)

This indicates that the reaction conditions are generally applicable. Gel analyses of the amplified exons and *mccB*5-6 of healthy individual LZ are depicted in Figure 4.7. Clear amplicons are visible on the gel analyses indicating successful amplification of the regions. Smears are visible in lanes 2, 3, 4, 5 and 6, suggesting excess gDNA. A faint band of <100 bp was visible in lane 2. Inspite of this amplicons of the appropriate size (2179 bp) were obtained. Gel analyses of CVH amplicons are not shown.

The amplicons of both individuals were gel extracted and sequenced in order to determine the sequence of the amplified regions. These sequence analyses served as a verification of the success of amplification and sequencing. Sequences were analysed using Chromas and Vector NTI software (Section 3.1.4.).

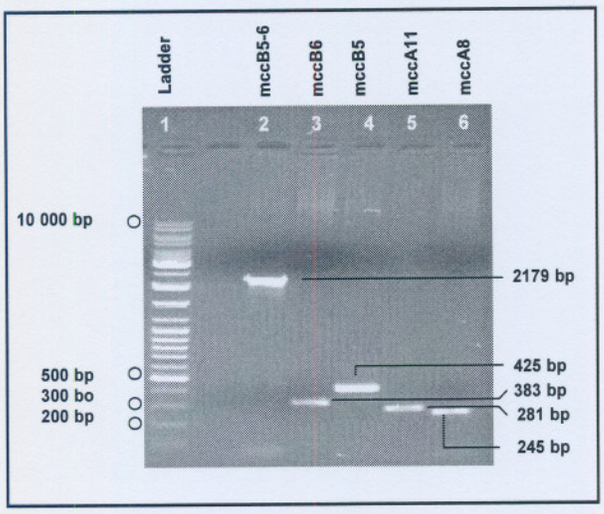


FIGURE 4.7: 1% Agarose gel analyses of the amplified exons from LZ buffy coat gDNA. Each lane consists of 5µl of a 50 µl sample. Lanes 1) DNA marker (O'GeneRuler cat no. SM 1173,Fermentas).; 2) *mccB*5-6; 3) *mccB*6; 4); *mccB*5 5) *mccA* 11and 6) *mccA*8.

4.2.3 Sequence analyses

The amplified exons *mccA*8, *mccA*11, *mccB*5, *mccB*6 and *mccB*5-6 of the three individuals CFC, LZ and CVH were sequenced. The sequence chromatograms were critically analysed for any ambiguities. Alignments of the exons with each other as with reference sequences were done. Since none of these individuals are MCC deficient, no mutations or polymorphisms were expected. The alignments showed no nucleic acid variation in the amplified exons. The amplification of *mccB*5-6 was successful with partial sequencing success. The lack of sequencing primers for the intron resulted in incomplete sequencing of *mccB*5-6. For the purpose of this study this sequencing is appropriate, since the presence of in5ac-1 could be determined.

```
AF310339          AATTTCAAGAACAGTTAGAGTCAGCACGGAGAGAAGCTAAGAAGTCTTTCAATGATGATGCTATGCTGATCGAGAAGTTTGTAGACACACCGAGGCATGT
AF310972          AATTTCAAGAACAGTTAGAGTCAGCACGGAGAGAAGCTAAGAAGTCTTTCAATGATGATGCTATGCTGATCGAGAAGTTTGTAGACACACCGAGGCATGT
Genomic mccA8     ----------------------------------------------------------------------------------------------------GCATGT
CFC_mccA_8        ----------------------------------------------------------------------------------------------------GCATGT
CVH_mccA_8        ----------------------------------------------------------------------------------------------------GCATGT
LZ_mccA_8         ----------------------------------------------------------------------------------------------------GCATGT

AF310339          AGAAGTCCAGGTGTTTGGTGATCACCATGGCAATGCTGTGTACTTGTTTGAAAGAGACTGTAGTGTGCAGAGGCGACATCAGAAGATCATTGAGGAGGCC
AF310972          AGAAGTCCAGGTGTTTGGTGATCACCATGGCAATGCTGTGTACTTGTTTGAAAGAGACTGTAGTGTGCAGAGGCGACATCAGAAGATCATTGAGGAGGCC
GenomicMCCA8      AGAAGTCCAGGTGTTTGGTGATCACCATGGCAATGCTGTGTACTTGTTTGAAAGAGACTGTAGTGTGCAGAGGCGACATCAGAAGATCATTGAGGAGGCC
CFC_MCCA_8        AGAAGTCCAGGTGTTTGGTGATCACCATGGCAATGCTGTGTACTTGTTTGAAAGAGACTGTAGTGTGCAGAGGCGACATCAGAAGATCATTGAGGAGGCC
CVH_MCCA_8        AGAAGTCCAGGTGTTTGGTGATCACCATGGCAATGCTGTGTACTTGTTTGAAAGAGACTGTAGTGTGCAGAGGCGACATCAGAAGATCATTGAGGAGGCC
LZ_MCCA_8         AGAAGTCCAGGTGTTTGGTGATCACCATGGCAATGCTGTGTACTTGTTTGAAAGAGACTGTAGTGTGCAGAGGCGACATCAGAAGATCATTGAGGAGGCC

AF310339          CCAGCGCCTGGTATTAAATCTGAAGTAAGAAAAAAGCTGGGAGAAGCTGCAGTCAGAGCTGCTAAAGCTGTAAATTATGTTGGAGCAGGGACTGTGGAGT
AF310972          CCAGCGCCTGGTATTAAATCTGAAGTAAGAAAAAAGCTGGGAGAAGCTGCAGTCAGAGCTGCTAAAGCTGTAAATTATGTTGGAGCAGGGACTGTGGAGT
GenomicMCCA_8     CCAGCG----------------------------------------------------------------------------------------------
CFC_MCCA_8        CCAGCG----------------------------------------------------------------------------------------------
CVH_MCCA8         CCAGCG----------------------------------------------------------------------------------------------
LZ_MCCA_8         CCAGCG----------------------------------------------------------------------------------------------
```

FIGURE 4.8: Sequence alignment of a published *mccA* sequences with exon *mccA*8 of three individuals. CFC represents amplicons from cultured fibroblast cells where CVH and LZ are amplicons from gDNA of buffy coat. Genomic *mccA*8; Ensembl ENSG00000078070

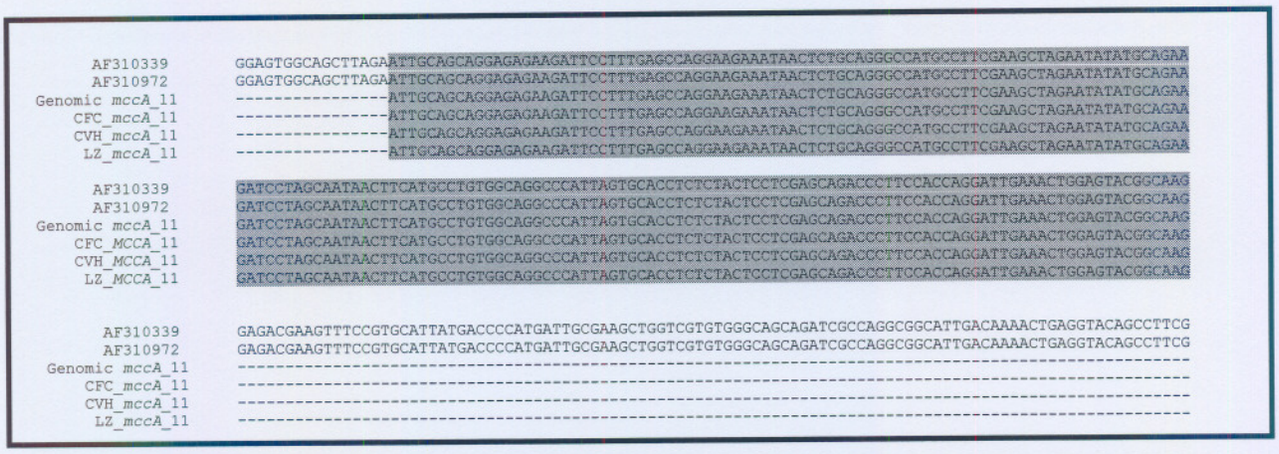


FIGURE 4.9: Sequence alignment of a published *mccA* sequence with exon *mccA*11 of three individuals. CFC represents amplicons from cultured fibroblast cells where CVH and LZ are amplicons from gDNA of buffy coat. Genomic *mccA*11; Ensembl ENSG00000078070

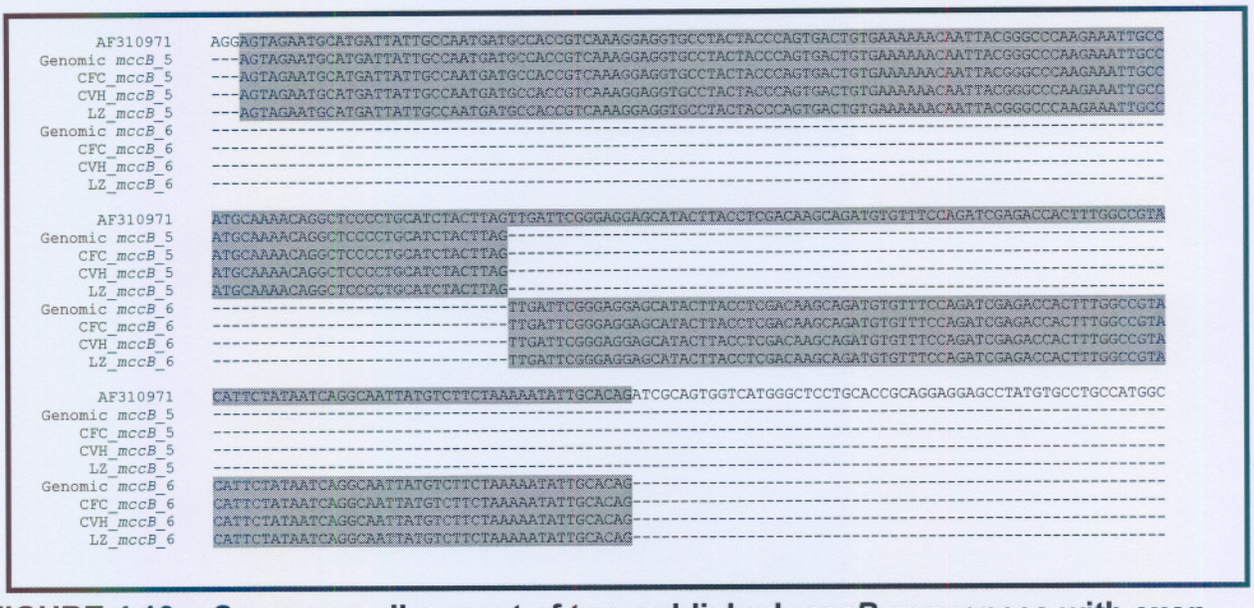


FIGURE 4.10: Sequence alignment of two published *mccB* sequences with exon *mccB*5 and exon *mccB*6 of three individuals. CFC represents amplicons from cultured fibroblast cells where CVH and LZ are amplicons from gDNA of buffy coat; Genomic *mccB*5, *mccB*6 and *mccB*5-6; Ensembl ENSG00000131844

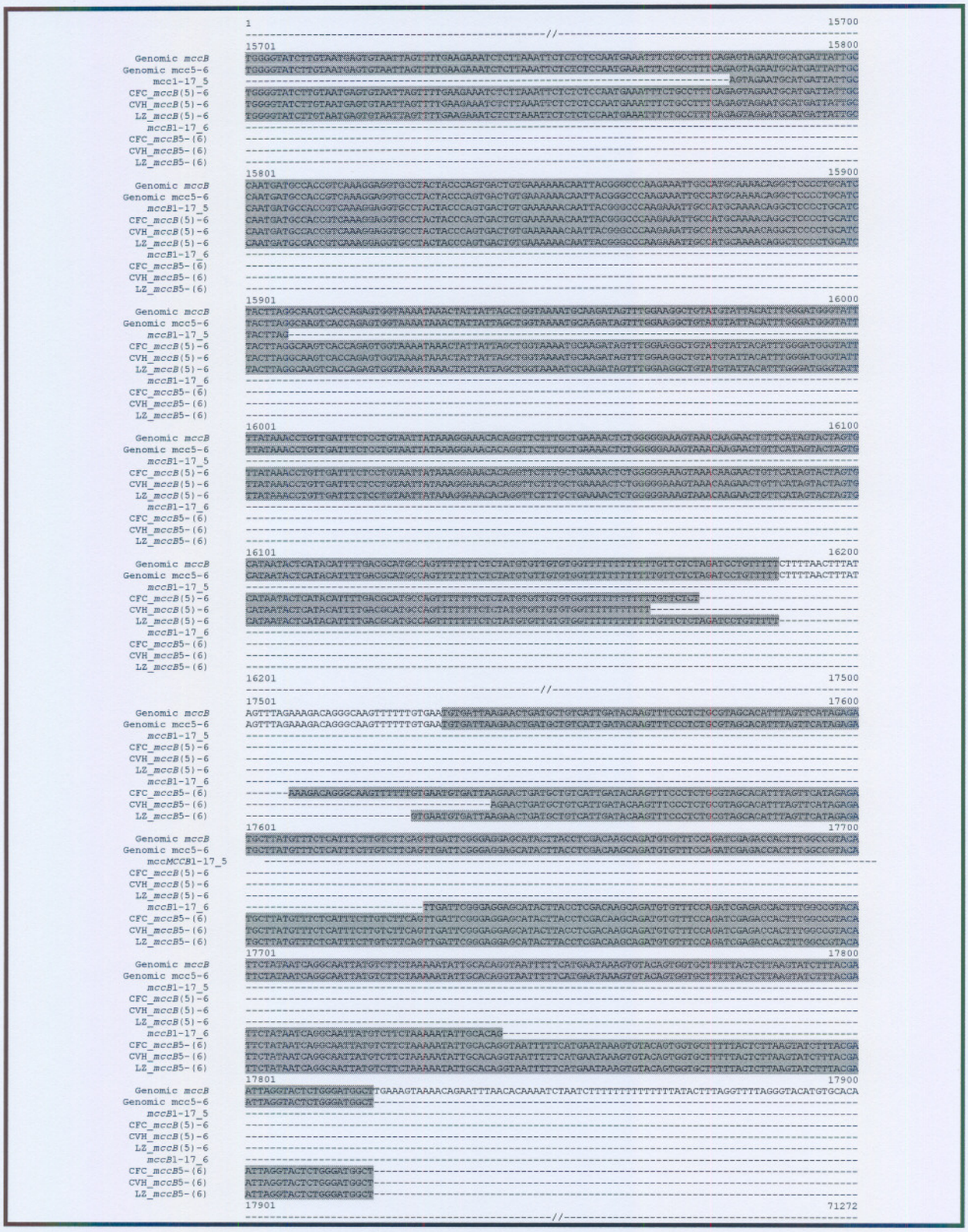


FIGURE 4.11: Sequence alignment of two published *mccB* sequences with exon *mccB*5-6 of three individuals. CFC represents amplicons from cultured fibroblast cells where CVH and LZ are amplicons from gDNA of buffy coat Genomic *mccB* (Ensembl ENSG00000131844) and Genomic *mccB*5-6 is the *mccB*5-6 region of Ensembl ENSG00000131844. *mccB*1-17_5 and *mccB*1-17_6 exons 5 and 6 from AF310971

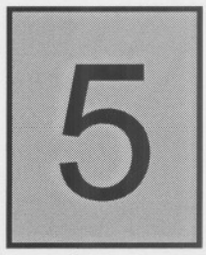
4.3 SUMMARY

Genomic DNA was successfully isolated using Flexigene® gDNA isolation kit from cultured fibroblast cells of three individuals (MS, ZL and CFC, see Figure 4.3 (a) Section 4.2.1) as well as from buffy coats of another three individuals (EVD, LZ and CVH, see Figure 4.3 (b) Section 4.2.1). Quantification of the isolated gDNA showed that the expected yield of 25-35 µg (Flexigene® Handbook, February 2003) corresponded with the resulted yield, approximately 40 µg. Good quality gDNA were obtained. The yields of gDNA isolated from buffy coats were less than those isolated from cultured fibroblast cells. The optimisation of gDNA isolation was a small problem. The number of cells used for the extraction resulted in the corresponding yield expected. The A_{260}/A_{280} ratio was 1,5 which indicated protein contamination. A better yield and A_{260}/A_{280} ratio wasobtained when an extra centrifugation step was inserted to pellet all the unlysed cells and so prevented interference of unlysed cells following cell lyses. The extra step was also used to isolate gDNA from buffy coat, the same approach was used (see appendix H). Although good quality gDNA were isolated from the buffy coats, the obtained yield did not correspond to the expected yield. According to the manufacturer's guidelines the expected yield of gDNA isolated from 1 ml buffy coat is approximately 220 µg (Flexigene® Handbook, February 2003). An average of 15 % (33 µg) gDNA was obtained from 1 ml buffy coat. Good quality gDNA was obtained. Amplification of the selected exons *mccA*8, *mccA*11, *mccB*5, *mccB*6 as well as genomic region *mccB*5-6 were done using gDNA isolated form cultured fibroblasts and gDNA isolated from LZ and CVH buffy coats (Figure 4.7). However, to optimally amplify exon *mccA*11 temperature and $MgCl_2$ adjustment had to be done. Optimal amplification of genomic region *mccB*5-6 needed temperature optimisation (see Section 4.2.2). Good sequences were obtained from these amplicons. Sequencing of the amplicons was excellent and sequence analyses indicated no mutations in any of the amplified regions of the individuals (CFC, LZ and CVH). No previously identified mutations for *mccA* and *mccB* as described in the literature (Baumgartner *et al.,* 2001; Gallardo *et al.,* 2001; Holzinger *et al.,* 2001, Dantas *et al.,* 2005) were detected in any of the individuals. Sequence alignments showed similarity between the amplified products of the different individuals and to the reference sequences (Figures 4.8, 4.9, 4.10, 4.11). None of the individuals were associated with MCC deficiency and therefore no mutations in the MCC genes (*mccA* and *mccB*) were expected. With a lack of sequencing primers for sequencing intron 5 of *mccB*5-6, only part of *mccB*5-6 could be sequenced. For the purpose of this study, only the exons and the intro-exon and exon-intron junctions needed to be sequenced. The particular

important the region includes the nucleic acid in position in5ac-1, since this position is associated with a mutation detected in a MCC deficient Caucasian individual.

In conclusion the results make, it appropriate to state that the implementation of the molecular characterisation of the chosen exons from the 19 exons of *mccA* (*mccA*8 and *mccA*11) and the 17 exons of *mccB* (*mccB*5 and *mccB*6) were successfully done on the genomic level. The implemented techniques can now be used to characterize *mccA* and *mccB* of MCC deficient individuals on a genomic basis. This can be done to confirm the results obtained from the amplification of *mccA* and *mccB* mature mRNA transcripts.

Chapter Five



Molecular characterisation of MCC of a South African MCC deficient patient

The incidence of MCC deficiency in the RSA is unknown, partly due to a lack of a comprehensive NBS programmes is largely responsible for this. The clinical symptoms of MCC deficiency vary from asymptomatic to severe neurological impairment. Patients often experience exhaustion, muscle weakness after excise and episodes of total loss of energy. Since these symptoms are associated with many illnesses IEMs are usually not considered initially when adults are involved. The patient (HGS, age 48) is an example of individuals that live their lives with an IEM without being aware of it. Patient HGS experienced 20 years of severe muscle cramps, chronic expenditure of energy, neuropathy and hepatomegaly. After many pathological tests, MCC deficiency was diagnosed biochemicaly. Elevated levels of 3-hydroxyisovaleric acid and 3-methylcrotonylglycine excreted in the urine were detected by GS/MS analyses. This patient (HGS) is the first individual diagnosed suffering from MCC deficiency in the RSA. Many IEMs have been molecularly studied. Molecular characterisation is often done to complement and extend MS/MS based NBS programmes. This improve the understanding of the disease and is needed for genetic counselling programmes that are part of NBS programmes (Gibson *et al.,* 1998; Naylor and Chace, 1999; Smith *et al.,* 2000; Roscher *et al.,* 2000; Wilcken *et al.,* 2000).

This chapter attempts to illustrate that molecular characterisation of MCC deficiency as presented in Chapters 4 and 5 is feasable. Four exons (*mccA*8, *mccA*11, *mccB*5 and *mccB*6) and genomic region *mccB*5-intron5-6-*mccB*6 were chosen for amplification. These exons were selected because most published mutations within this region are associated with Caucasian individuals. Since the South African patient (HGS) is of Caucasian origin, are these exons are a good starting point for the molecular characterisation of MCC of the MCC deficient patient. Since theres no guarantee that the disease causing mutation is present in the selected exons, it is appropriate to characterize the entire ORF of both MCC subunits. The ORF of the alpha subunit consists of 19 exons were the ORF of the smaller beta subunit consists of 17 exons.

5.1 MATERIALS AND METHODS

The methods to characterize MCC of HGS are those implemented and described in the previous two chapters (Chapters 3 and 4). A primary skin fibroblast cell culture of the index patient (Section 3.1.1.1, and blood samples, collected in heparin coated tubes to isolate buffy coats (Section 4.1.1.2). From the cultured cells nucleic acids gDNA and/or totoal RNA, were isolated using methods as described in previous Chapters 3 and 4. gDNA was also isolated from buffy coat (Figure 5.1(a)). The isolated gDNA from buffy coat and cultured fibroblast cells were used as template for the amplification of selected exons. From the isolated total RNA, cDNA was synthesized and the transcripts of both genes (*mccA* and *mccB*) were amplified using a two step RT-PCR approach. The amplified exons and transcripts (*mccA* and *mccB*) were gel extracted and sequenced. The sequences were critically analysed in order to identify the possible disease causing mutation.

Primary fibroblast cell cultures were established from a skin biopsy of HGS as described in Section 3.1.1 by a commercial genetic consultation laboratory GENEPATH, Jakaranda Hospital, Sunnyside, Pretoria. Upon receipts of the established primary fibroblast cells in our laboratory were split 1:4 for 5 passages. After one 25 cm^2 flasks reached 80 – 100 % confluency, it was used for the isolation of nucleic acids, either gDNA or total RNA as described in Section 4.1.1 and 3.1.2 respectively.

5.2 RESULTS AND DISCUSSION

5.2.1 gDNA and total RNA isolation

The same protocols and extraction kits previously standardized were used for the isolation of gDNA (Section 4.1.1) and total RNA form cultured fibroblast cells as described in and Section 3.1.2. Isolated gDNA was spectrophotometrically analysed using Nanodrop® as well as on a 1 % agarose gel (Figure 5.1a). Isolated total RNA was also spectrophotometrically analysed using Nanodrop® as well as analysed on a 1,2 % denaturing formaldehyde gel (Figure 5.1 b) or a 1 % agarose gel electrophoresis. A summary of the concentration and yield of the spectrophotometric quantified gDNA and total RNA isolated form HGS cultured fibroblast cells are listed in Tables 5.1 and 5.2. Good quality gDNA was isolated from cultured fibroblasts and buffy coat with an A_{260}/A_{280} ration of 1.88 The average yield of gDNA from cultured fibroblasts was 43,3 µg and from buffy coat it was 24,4 µg. The expected yield of isolated gDNA from 1-3 x 10^6 cells per 25 cm^2 is 25 - 35 µg and from 1 ml buffy coats 110-260 µg (Flexigene® Handbook, February 2003).

TABLE 5.1: Quantification of gDNA isolated from cultured fibroblast cells of a MCC deficient patient (HGS)

Starting material	Nucleic Acid	Method	Concentration ng/µl	Total yield (µg) in 200 µl 10 mM Tris-Cl pH8,5 (EB)buffer	Ratio A_{260} / A_{280}
Buffy coat	DNA	Flexigene®	121,76	24,4	1,88
Fibroblasts	DNA	Flexigene®	216,43	43,3	1,88

The yield of the isolated gDNA from cultured fibroblasts exceeded the amount of gDNA expected with approximately 25 % gDNA isolated. The yield of the gDNA isolated from buffy coat was only 22 % of the expected yield for gDNA isolated from 1 ml buffy coat. Although the yield was low, the A_{260}/A_{280} of the isolated gDNA was 1,88. The purity of the isolated gDNA was expected and since good quality gDNA was isolated, PCRs were prepared.

TABLE 5.2: Quantification of total RNA isolated from cultured fibroblast cells of a MCC deficient patient (HGS)

Starting material	Nucleic Acid	Method	Concentration ng/µl	Total yield (µg) in 50 µl RNase free water	Ratio A_{260} / A_{280}
Fibroblasts	RNA	RNeasy®	202,4	10,0	2,12
Fibroblasts	RNA	Qiazol®	2703,78	135,2	2,01

The total RNA preparations were successful. Gel analyses depicted in Figure 5.1b, shows intact total RNA. Two bands 28S and 18S representing rRNA subunits are present in a ratio of 2:1, an indication of good quality total RNA was isolated. This prepared total RNA can be used for cDNA synthesis and PCR can follow.

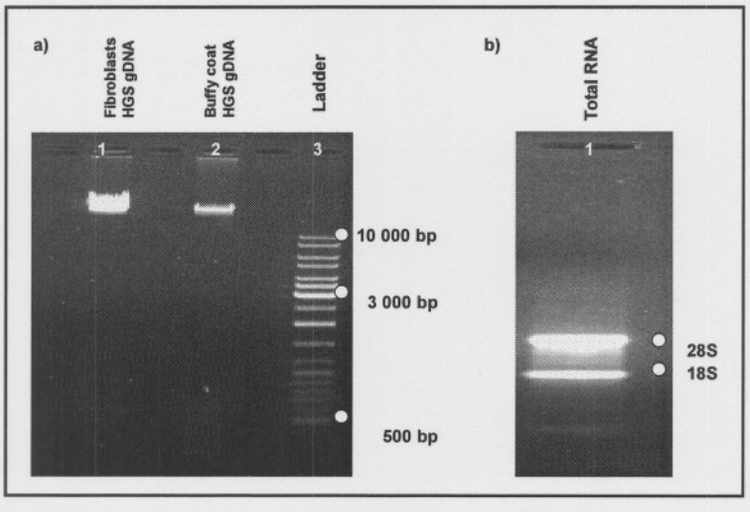


FIGURE 5.1: a) 1% Agarose gel analyses of gDNA isolated from HGS cultured fibroblast cells and buffy coat; b) 1,2 % Formamide agarose gel analyses of total RNA isolated from HGS cultured fibroblast cells.
Lanes: a1) 5 µl of a 200 µl HGS gDNA sample isolated from cultured fibroblast cells; a2) 5 µl of a 200 µl HGS gDNA sample isolated from buffy coat; a3) Ladder O'GeneRuler (cat no. SM 1173 Fermentas) and b1) 5 µl of a 40 µl HGS total RNA preparation from cultured fibroblast cells.

The quality gDNA isolated from HGS cultured fibroblast cells was equivalent to the quality gDNA preparations from CFC cultured fibroblast cells (Chapter 4). Although the yield obtained from HGS is approximately 10 µg more than that obtained from CFC. Good quality gDNA wasprepared from buffy coats of CVH, LZ, EVD and HGS with an average A_{260} / A_{280} ratio of 1,8. Although good quality gDNA wasisolated was an average of only 22 % of the expected yield obtained.

5.2.2 Amplification and sequence analyses of *mccA*8, *mccA*11, *mccB*5, *mccB*6 and *mccB*5-6

The selection of the specific regions to be amplified was based on published data (Baumgartner *et al.,* 2001; Gallardo *et al.,* 2001; Holzinger *et al.,* 2001, Dantas *et al.,* 2005). The ethnic origin of the MCC deficient individuals was correlated to the mutation present within the specific exons and regions of the MCC genes (*mccA* and *mccB*). Since no molecular information regarding MCC deficiency in SA is known, the identification of previously described mutations of individuals with the same ethnic origin is a good starting point in the molecular characterisation of MCC deficiency in SA. The exons with mutations reported for Caucasians were chosen for amplification. Since HGS is of Caucasian origin. Exons that were selected first were *mccA*8, *mccA*11, *mccB*5, *mccB*6 and the genomic region *mccB*5-intron5-6-*mccB*6. Some mutations associated with other ethnic groups were also included. These mutations were present in MCC deficient individuals with mild clinical effects as in the case of HGS (SA MCC deficient individual). Tables 5.3 and 5.4 are extracted from Tables 2.2 and 2.3 Section 2.6, Chapter 2 to highlight the mutations of relevance to the SA MCC deficient Caucasian individual (HGS).

Amplification of the selected exons followed. Specific annealing temperatures and $MgCl_2$ concentrations for the amplification of each exon are given in Table 4.4, Section 4.2.2. The amplicons were analysed on a 1 % TAE agarose gel. Amplicons for *mccA*8, *mccA*11, *mccB*5 and *mccB*6 were clearly visible on the gels depicted in Figure 5.2. The smear > 10 000 bp indicated that the gel was overloaded. In lanes 1, 3 and 5 the band <100 bp (primer dimers) is an indication of an excess of primers. However, the yield of *mccB*5-6 spanning *mccB*5 and *mccB*6 and the intron 5-6 between then, was low and there was a smear just below it (Figure 5.2 a lane 1). Therefore, this amplicon (*mccB*5-6) was gel extracted (Section 3.1.4, Appendix M) before sequencing.

Both strands of each amplicon were sequenced. Chromatograms were analysed and the sequence of the coding and non-coding strands were aligned with themselfes and the same reference sequences used in Chapters 3 and 4. From the alignments and chromatograms, no mutations were observed in HGS *mccA*8, *mccA*11, *mccB*5, *mccB*6 of *mccB*5-6.

Table 5.3: Mutations of the alpha subunit (*mccA*) relevant to the SA MCC deficient Caucasian individual (HGS)

Mutation	Exon	Nucleotide change	Clinical outcome	Population group	Reference
A291V Missense	8	872C→T	-	Australian	Dantas *et al.*, 2005
A289V	8	866C→T	Mild	American	Baumgartner *et al.*, 2001
Q421AfsX10 Frameshift	11	1263dupG	Mild	Swedish	Dantas *et al.*, 2005
R385S Missense	11	1155A→C	Severe	German	Baumgartner *et al.*, 2001; Dantas *et al.*, 2005
R385S	11	1155A→C	Severe	USA	Gallardo *et al.*, 2001
Q421fs(+1)	11	1264insG	Mild	Swedish/ American	Baumgartner *et al.*, 2001

Table 5.4: Mutations of the beta subunit (*mccB*) relevant to the SA MCC deficient Caucasian individual (HGS)

Mutation	Exon	Nucleotide change	Clinical outcome	Population group	Reference
C167R Missense	5	499T→C	-	Spain	Gallardo *et al.*, 2001
R155Q Missense	5	464G→A	Mild	Vietnamese	Baumgartner *et al.*, 2001
R155W Missense	5	463C→T	Mild	Brazilian	Dantas *et al.*, 2005
R155Q Missense	5	464G→A	-	Turkish	Dantas *et al.*, 2005
Q157X Nonsense	5	469C→T	-	Turkish	Dantas *et al.*, 2005
T139_G143→RWVPGEfsX35 Frame shift	5	416_427del12ins16	-	Turkish	Dantas *et al.*, 2005
In5ac-1G→A Splice site		In5ac-1G→A	Mild	Dutch	Baumgartner *et al.*, 2001
D172fs Frame shift	6	517insT	-	USA	Gallardo *et al.*, 2001
R193C Missense	6	577C→T	Mild	Dutch	Baumgartner *et al.*, 2001
S173L Missense	6	518C→T	Severe	Turkish	Baumgartner *et al.*, 2001
H190Y Missense	6	568C→T	Mild	Turkish	Dantas *et al.*, 2005
S173FfsX25 Frame shift	6	517dupT	Mild	Swiss / Mennonite Swiss American	Baumgartner *et al.*, 2001 Dantas *et al.*, 2005

Alignment results of each amplified genomic region are depicted in Figures 5.3; 5.4; 5.9. Chromatograms of the position of previously documented mutations in MCC deficient patients are also depicted to confirm that the published mutations were not present (Figures 5.5; 5.6; 5.7; 5.8; 5.10)

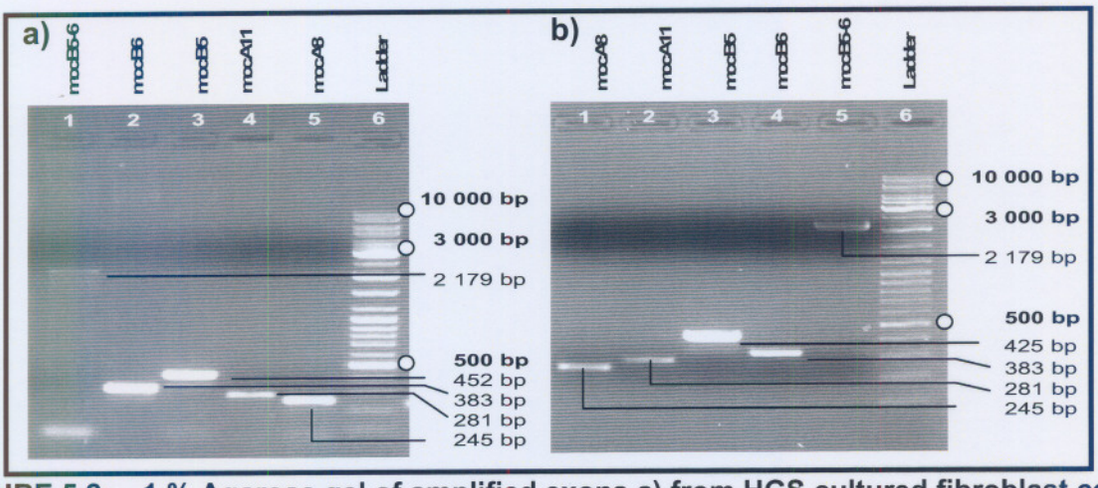


FIGURE 5.2: 1 % Agarose gel of amplified exons a) from HGS cultured fibroblast cells; b) form HGS buffy coat. Lanes a1) *mccB*5-6, a2) *mccB*6, a3) *mccB*5, a4) *mccA*11, a5) *mccA*8, a6) Ladder O'GeneRuler (cat no. SM 1173 Fermentas) b1) *mccA*8, b2) *mccA*11, b3) *mccB*5, b4) *mccB*6, b5) *mccB*6 and Ladder O'GeneRuler (cat no. SM 1173 Fermentas).

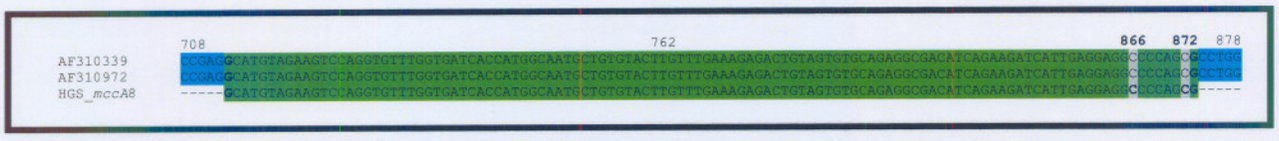


FIGURE 5.3: Sequence alignment of HGS*mccA*8. AF310339 and AF310972 are *mccA* mature mRNA transcript reference sequences; Green regions are exon *mccA*8; bold nucleic acids 866 and 872 are nucleic acid position in the mature mRNA transcript of previously described mutations. The blue regions represents a parts of the flanking exons 7 and 9.

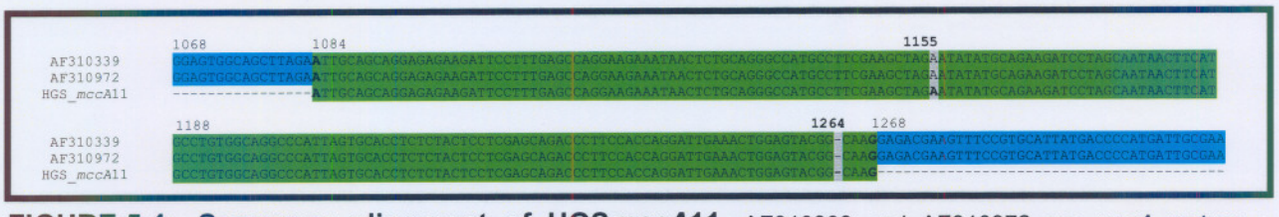


FIGURE 5.4: Sequence alignment of HGS*mccA*11. AF310339 and AF310972 are *mccA* mature mRNA transcript reference sequences; Green regions are exon *mccA*11; bold nucleic acids 1155 and 1264 are nucleic acid position in the mature mRNA transcript of previously described mutations. The blue regions represent parts of the flanking exons 10 and 12.

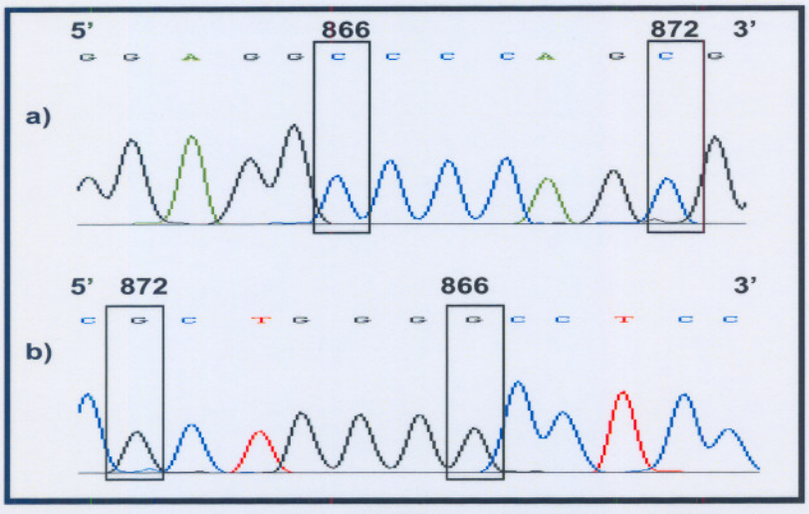


FIGURE 5.5: Sequence chromatograms of HGS*mccA*8 (866 and 872). a) cytosines (866 and 872) of the coding strand (5'-3'), and b) guanines (872 and 866 of the non-coding strand (5'-3').

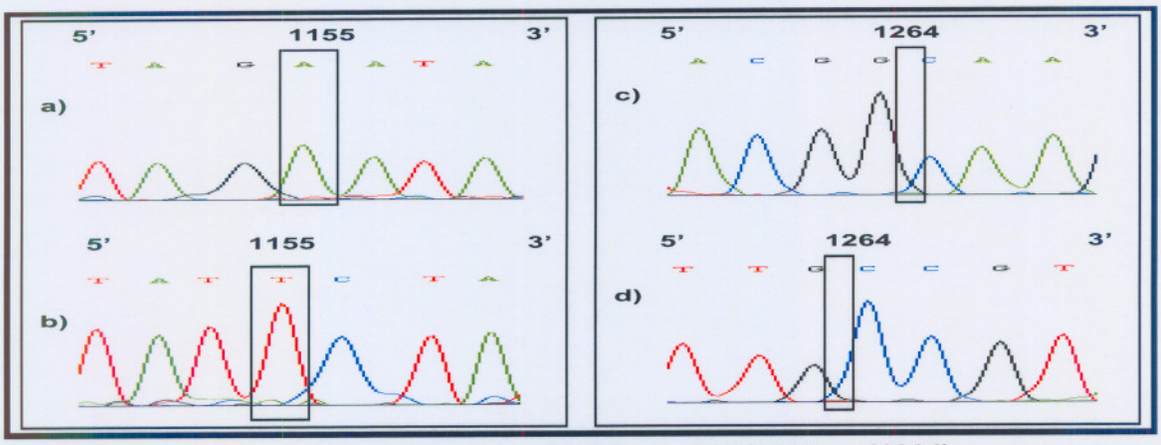


FIGURE 5.6: Sequence chromatograms of HGS*mccA*11 (1155 and1264). a) Adenine (1155) and c) position 1264 of the coding strand (5'-3'), and b) thymine (1155) and d)position 1264 of the non-coding strand (5'-3').

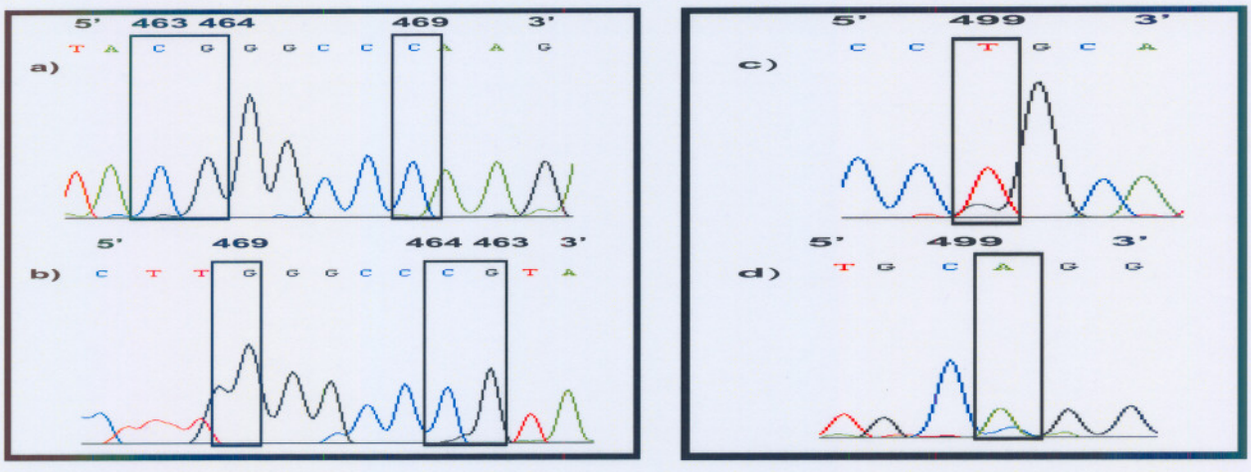


FIGURE 5.7: Sequence chromatograms of HGS*mccB*5 and HGS*mccB*6 (463, 464, 467, and 499) a) Cytosines (463 and 469) and the guanine (464) of the coding strand (5'-3') and b) guanines (463 and 469) and the cytosine (464) of the non-coding strand (5'-3'); c) Thymine (499) of the coding strand and d) adenine (499) of the non-coding strand.

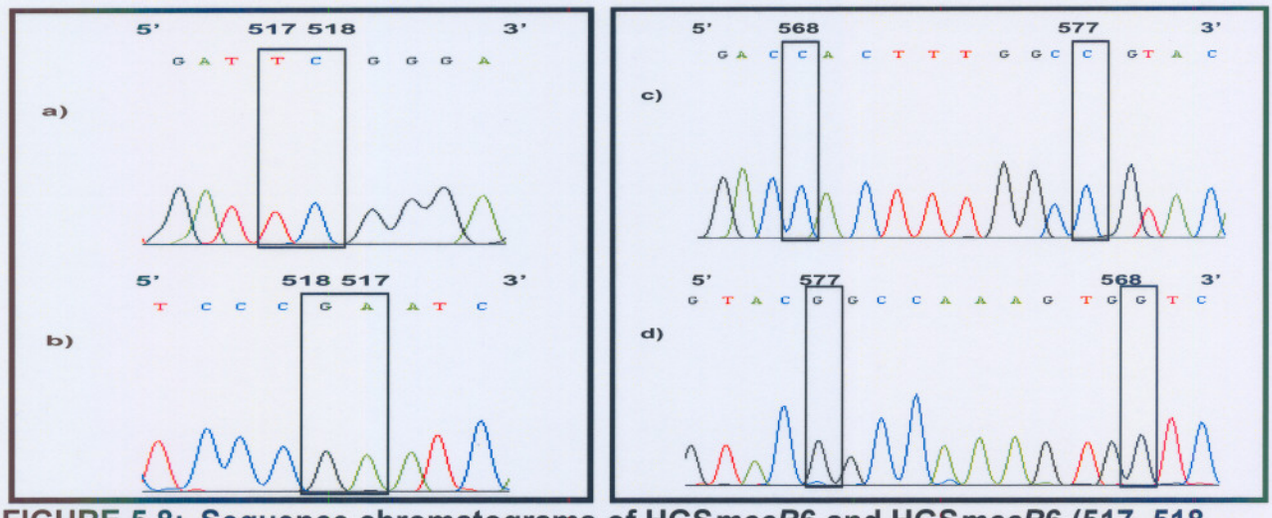


FIGURE 5.8: Sequence chromatograms of HGS*mccB*6 and HGS*mccB*6 (517, 518, 568, and 577) a) Thymine (517) and cytosine (518) of the coding strand (5'-3') and b) adenine (517) and guanine (518) of the non-coding strand (5'-3'); c) cytosines (568 and 577) of the coding strand and d) guanines (568 and 577) of the non-coding strand.

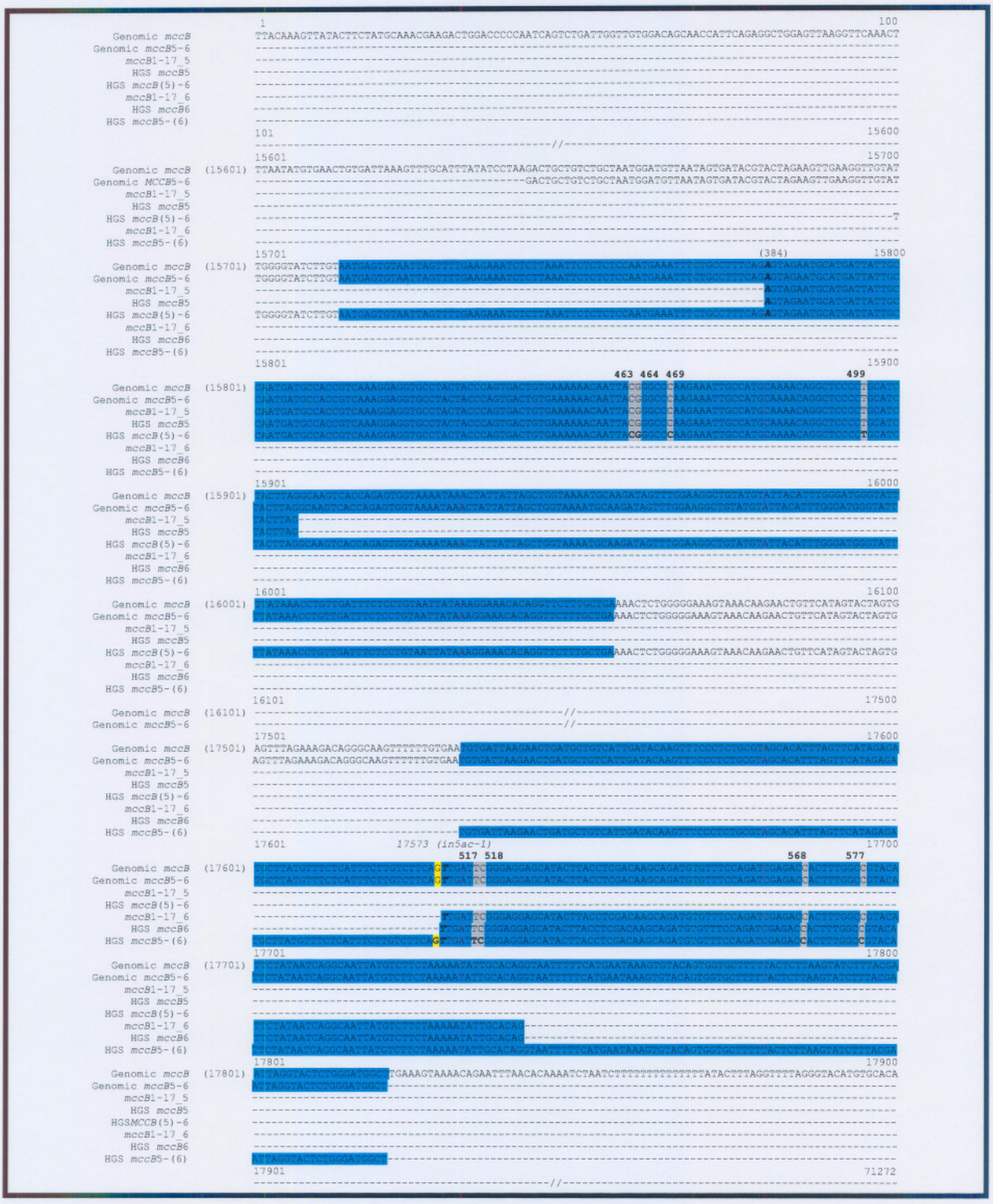


FIGURE 5.9: Sequence alignment of HGS*mccB*5, HGS*mccB*6 and HGS*mccB*5-6.
Genomic *mccB* is the genomic sequence Ensembl ENSG00000131844 of the beta subunit; Grey and bold nucleic acids 463, 464, 469, 499, 517, 518, 568, and 577 are nucleic acid position in the *mccB* mature mRNA transcript of previously described mutations. Yellow and bold is the nucleic acid Blue regions is exon *mccB*5, *mccB*6 and the sequences region of *mccB*5-6; and bold nucleic 17573 (in5ac-1) of a previously documented mutation in the acceptor site (3') of intron 5. Genomic *mccB*5-6 is the exon5-intron5-6-exon6 from genomic sequence Ensembl ENSG00000131844

The entire intron sequence was not determined, due to a lack of intron specific sequence primers. Since the sequences of the genomic region (*mccB*5-6) included position 17573 (in5ac-1), it was appropriate not to sequence the entire intron but using only the PCR primers for sequencing (Table 4.1).

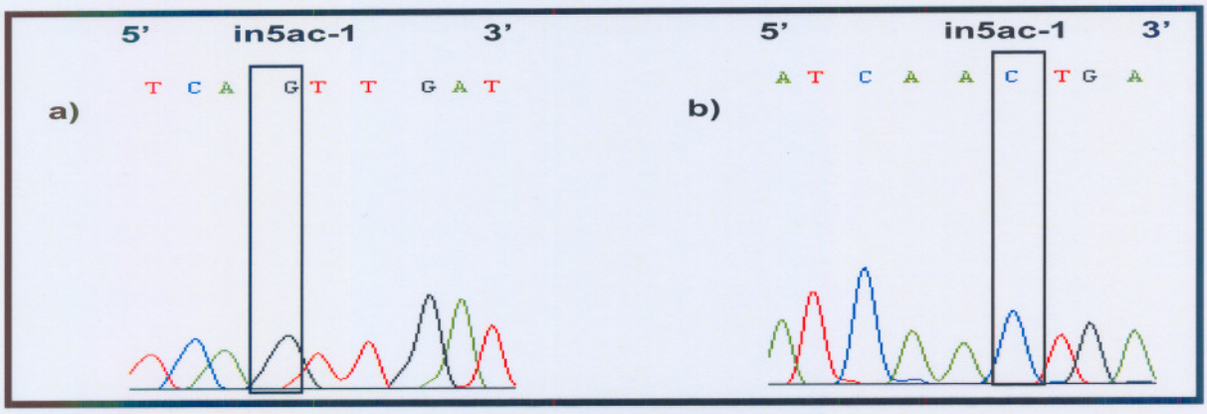


FIGURE 5.10: Sequence chromatograms of HGS*mccB*5-6 (in5ac-1) a) guanine (in5ac-1) of the coding strand (5'-3') and b) cytosine (in5ac-1) of the non-coding strand (5'-3')

5.2.3 RT-PCR Amplification and sequence analyses of HGS*mccA* and HGS*mccB* mature mRNA Transcripts

Since no mutations were identified in the selected exon sequence, the next step was to RT-PCR amplify the mature mRNA transcripts of both MCC subunits (*mccA* and *mccB*) of HGS and sequence the amplicons. In this way the sequence of all 19 exons of HGS*mccA* and the 17 exons of HGS*mccB* can be determined and the amino acid sequence deduced to analyse for mutations.

Isolated total RNA was used as template for cDNA syntheses (Section 3.2.2). Good quality total RNA was isolated using the Qiazol® lyses reagent (Table 5.2). The mature mRNA transcripts of both subunits *mccA* and *mccB* were amplified using the implemented methods described in Chapter 3. Total RNA isolated from HGS cultured fibroblasts were used. During the amplification of the subunits, the housekeeping gene *GAPDH* was included as a positive control to verify the success of cDNA synthesis (Figure 5.12).

The MCC subunits of HGS (HGS*mccA* and HGS*mccB*) and *GAPDH* were successfully amplified. Amplicons of the expected size (2326 bp for HGS*mccA*, 1926 bp for HGS*mccB* and 226 bp for *GAPDH* were generated. However, multiple amplicons were obtained for HGS*mccA* and HGS*mccB*. HGS*mccA* had a strong band of approximately 2326 bp with several weaker bands of approximately 100-500 bp. The expected HGS*mccB* amplicons of 1926 bp was obtained, but the yield was low and several other amplicons were also generated.

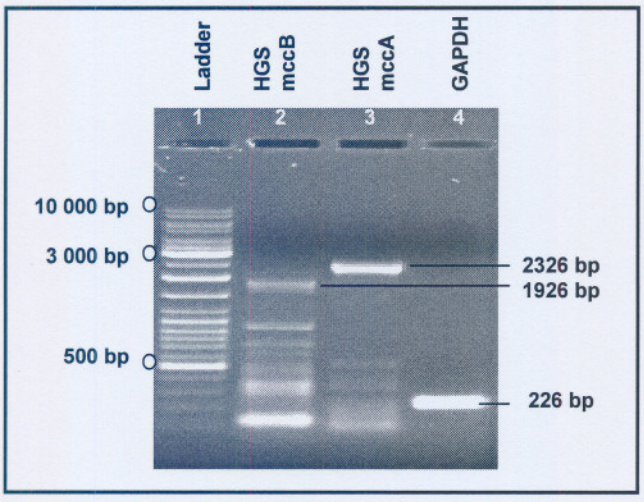


FIGURE 5.11: 1 % AGAROSE GEL ANALYSES OF HGS*mccA*, HGS*mccB* AND *GAPDH*
Lanes: 1) DNA marker (O'GeneRuler, (cat no. SM 1173 Fermentas); 2) amplified HGS*mccB*; 3) HGS*mccA* and 4) amplified *GAPDH*

The presence of the multiple bands was unexpected, since no non-specific amplicons were generated during the implementation and standardisation of the methods and protocols described in Chapter 3. The only variation that occured during the implementation of the molecular characterisation and the application of the implemented characterisation was the template used. Therefore, the multiple amplicons were thought to be specific for the MCC deficient individual, HGS. The amplicons of the approximately correct size were gel extracted and sequenced using primers listed in Tables 3.2 and 3.3 (Section 3.1.4.1). These primers used for sequencing are appropriate for sequencing the entire ORF of HGS*mccA* and HGS*mccB*, with overlapping coding and non-coding sequences, but in order to sequence both strands (coding and non-coding) entirely, should more primers be designed. It is expected that the entire ORF of both subunits will be sequenced, but with overlapping coding and non-coding sequences for both subunits. Chromatograms of each MCC subunit of HGS were analysed. The alpha subunit *mccA*, was entirely sequenced as in the case of CFC*mccA* (Chapter 3). Unfortunately, the beta subunit was not entirely sequenced. The region that includes exons 7 and 8 could not be sequenced. Since these exons were not included in the exon selection that was amplified on genomic level, it was not possible to analyse this regions (exons 7 and 8) of *mccB*. The sequences of the amplified exons as well as the sequences of the mature mRNA transcript were aligned with each other as well as with reference sequences taken from GenBank. These alignments are depicted in Figure 5.12 (HGS*mccA*) and Figure 5.14 (HGS*mccB*).

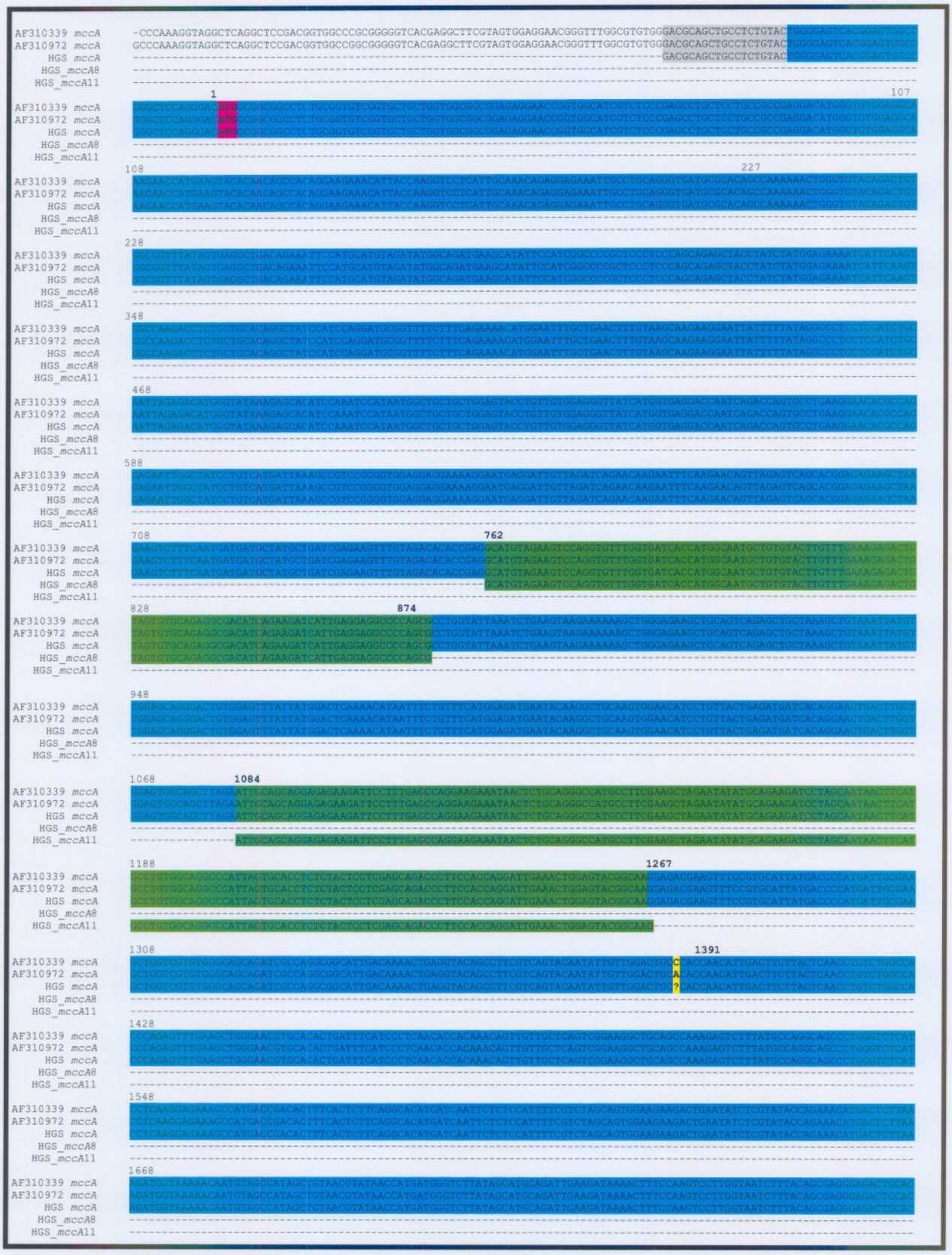


FIGURE 5.12: Alignment of HGS DNA sequences of exons *mccA*8 and *mccA*11 Purple; ATG initiation codon and TAA the termination or stop codon of the HGS*mccA* ORF. Grey: Primer region; Yellow: position 1391 A →C (can be A or C, see Figure 5.15); Green HGS*mccA*8 (762-874) and HGS*mccA*11 (1084-1267), exons that were sequenced on the gDNA.....CONTINUE

FIGURE 5.12: Alignment of HGS DNA sequences of exons *mccA*8 and *mccA*11 Purple; ATG initiation codon and TAA the termination or stop codon of the HGS*mccA* ORF. Grey: Primer region; Yellow: position 1391 A →C (can be A or C, see Figure 5.15); Green HGS*mccA*8 (762-874) and HGS*mccA*11 (1084-1267), exons that were sequenced on the gDNA

The alpha subunit (HGS*mccA*) was fully sequenced. Sequence analyses obtained from HGS*mccA* showed one possible SNP, nucleic acid change 1391A→C which results in the H464P amino acid change (GenBank). As depicted in Figure 5.15, is it not clear whether position 1391 of the HGS*mccA* ORF is an adenine or a cytosine. The possibility of a heterozygote should not be eliminated. Since two sequences (Figure 5.13) sequenced at different times shows the same resulted of two picks on the same position. The lack of a non-coding strand sequence hampers the identification the nucleotide in this position (1391). Therefore, it is recommended to design primers in order to sequence the non-coding strand that includes this region.

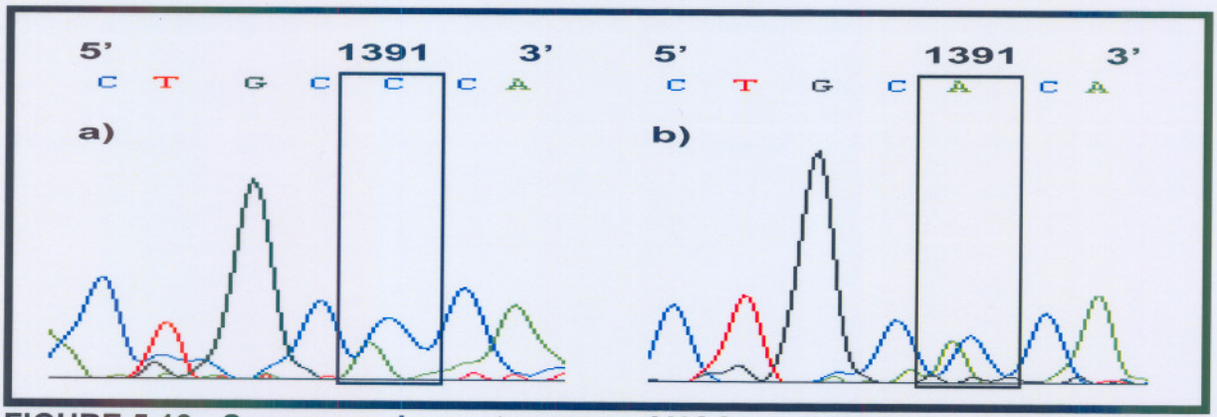


FIGURE 5.13: Sequence chromatograms of HGS*mccA* (1391 A→C (?)) a) cytocine (1391) of the coding strand (5'-3') and b) adenine (1391) of the coding strand (5'-3')

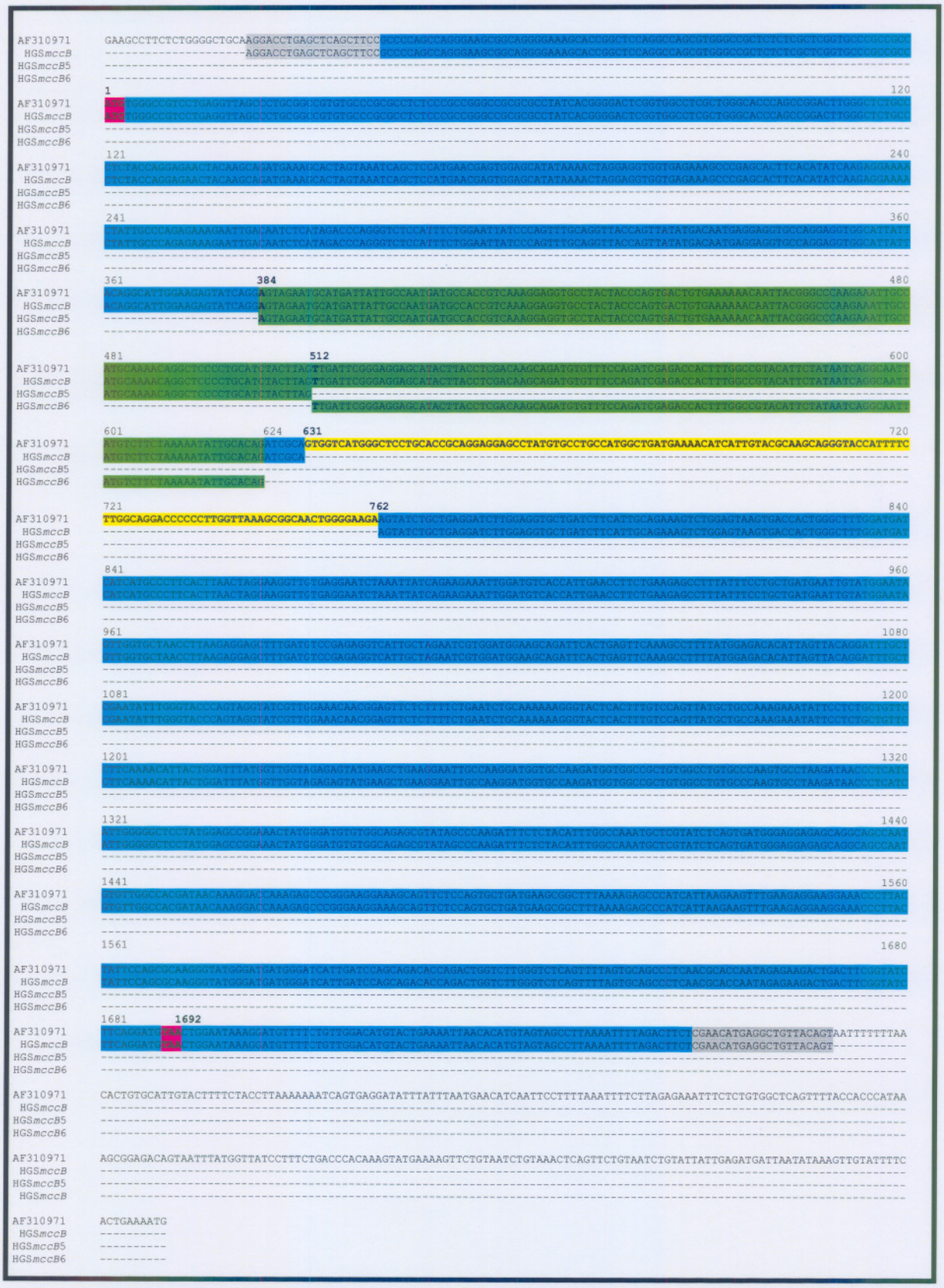


FIGURE 5.14: Alignment of HGS DNA sequences of exons *mccB*5 and *mccB*6 Purple; ATG the beginning and TAA the termination or stop of the HGS*mccB* ORF. Grey: Primer region; Green HGS*mccB*5 (384-512) and HGS*mccB*6 (513-624); Yellow, region of the HGS*mccB* ORF for which no sequence isavailable.

The alignments of the sequenced regions show no mutations identified in the genomic regions *mccA*8, *mccA*11, *mccB*5, *mccB*6 or *mccB*5-6. The entire ORF of HGS*mccA* was sequenced and aligned. One possible SNP was detected. The ORF of HGS*mccB* was only partly sequenced. No mutations were detected in the sequenced regions.

5.3 SUMMARY

Genomic DNA was successfully isolated using the Flexigene® genomic DNA isolation kit from cultured fibroblast cells as well as from buffy coat (Figure 5.1). Quantification of the isolated gDNA showed that the expected yield of 25-35 µg (Flexigene® Handbook, February 2003) corresponded with the yield that was obtained, approximately 40 µg. The yields of gDNA isolated from buffy coat were only 22 % of the expected yield. However, good quality gDNA was isolated from the buffy coat and PCR could be performed. Amplification of the selected exons *mccA*8, *mccA*11, *mccB*5, *mccB*6 as well as genomic region *mccB*5-6 (Figure 5.2) was completed using gDNA isolated form cultured fibroblast cells of HGS as well as gDNA isolated from buffy coat of HGS. Amplicons were sequenced. Good sequences were obtained and sequence analyses showed no variation in any of the amplified regions of HGS compared to MCC sequences from GenBank. No identified mutations for *mccA* and *mccB* as described in the literature for MCC deficient patients (Baumgartner *et al.,* 2001; Gallardo *et al.,* 2001; Holzinger *et al.,* 2001, Dantas *et al.,* 2005) were detected. As stated previously (Section 4.3), a lack of sequencing primers for sequencing intron 5 of *mccB*5-6, resulted in only partial *mccB*5-6 sequence. For the purpose of this study only the exons together with the intron-exon and exon-intron junctions should be sequenced in5ac-1, since this position is associated with a mutation detected in a MCC deficient Caucasian individual (Baumgartner *et al.,* 2001).

To summarize the results obtained; the molecular characterisation of the specific exons chosen from the 19 exons of *mccA* (*mccA*8 and *mccA*11) and the 17 exons of *mccB* (*mccB*5 and *mccB*6) were successful done on genomic level. However, no mutations were detected. Therefore, the next step was to isolate total RNA from HGS cultured fibroblasts in order to amplify the entire ORF of the MCC genes (*mccA* and *mccB*). This approach allows all 19 exons of *mccA* and 17 exons of *mccB* to be amplified simultaneously (Chapter 3).

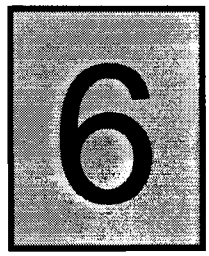
Good quality total RNA was isolated from cultured fibroblast cells using the Qiazol® reagent. To eliminate the interference of phenol and chloroform during RT-PCR the total RNA preparation was cleaned with RNeasy® columns using the cleanup protocol. Isolated total RNA was analysed on a 1 % agarose as well as 1,2 % formaldehyde denaturing agarose gel (Figure5.2b). ntact rRNA (28S and 18S) was present in an approximate ratio of 2:1 ratio (28S:18S). The isolated total RNA was used for first strand cDNA synthesis. The amplification of both

transcripts i.e. (HGS*mccA* and HGS*mccB*) of the MCC deficient patient, HGS, was done. The amplicons consists of multiple amplicons. The expected bands (*mccA* 2326 bp and *mccB* 1926 bp) were gel extracted and sequenced. The alpha subunit (HGS*mccA*) was fully sequenced, but HGS*mccB* was only partly sequenced. Sequence analyses obtained from HGS*mccA* showed only one possible SNP, nucleic acid change 1391A→C which results in the His464Pro amino acid change. Available sequences of the beta subunit HGS*mccB* was carefully analysed but no mutation was detected.

Some problems arose during the application of the implemented molecular characterisation of MCC in SA (Chapter 3 and 4). The multiple bands obtained from the HGS*mccA* and HGS*mccB* amplification could be due to non-specific or specific amplification. Non-specific amplification occurs when the primer annealing temperature or the $MgCl_2$ concentration is not optimal. Another possibility is splice variants. However, only one amplicon is expected with an unexpected size. To rule out the possibility of non-specific amplification due to annealing temperature and the $MgCl_2$ concentration that is not optimal, temperature gradients and $MgCl_2$ gradients should be included. The other bands should also be sequenced to determine whether the amplified bands are specific with respect to the expected fragment sequence. The best solution would be to design primers in new regions or extend primers to be more specific for the region. Especially when primer sequences occurs in positions known to be a hotspot for mutations. With the lack of MCC deficient patients, it is difficult to determine whether the non-specific priming is due to non-optimal PCR conditions whether the amplified fragment pattern is specific to the individual (HGS) implicating that he has multiple priming regions for these primers. MCC genes of more individuals should be amplified to test the robustness of the primer sets.

To summarize this Chapter, nucleic acids were successfully isolated from HGS. Amplification and sequencing of the genomic regions (*mccA*8, *mccA*11, *mccB*5, *mccB*6, *mccB*5-6) were successful analysed. No mutations or SNPs were detected in these regions. The mature mRNA transcripts were RT-PCR amplified. The multiple band amplicons were unexpected since the implementation of the molecular characterisation of the mature mRNA transcripts (Chapter 3) did not result in multiple band amplicons. Sequence analyses were successful for HGS*mccA* where only one possible SNP (1391A →C) was identified. Incomplete sequences of HGS*mccB* resulted in partial characterisation of HGS*mccB*. No mutations or SNPs were identified in the sequenced regions (exon1-6 and 9-17) of HGS*mccB*.

Chapter Six



Conclusion

This chapter summarizes the results obtained during this study. The limitations that were experienced as well as some recommendations for future developments are also presented.

For RSA to catch up and contribute to the field of MCC research and serve people having this disease it is important that molecular approaches be implemented to complement the MS/MS based NBS programmes. Genetic screening is one of the most common extended programmes running in addition to MS based NBS worldwide (Romero *et al.,* 2005). As MCC deficiency is considered an emerging disease due to the frequency with which it is detected in NBS programmes, it is appropriate to study this disease extensively on molecular level. The enzyme MCC is comprised of two subunits, alpha (*mccA*) and beta (*mccB*). The genes encoding these subunits are well characterized in Europe and the USA (Baumgartner *et al.,* 2001; Gallardo *et al.,* 2001; Holzinger *et al.,* 2001; Dantas *et al.,* 2005; Stadler *et al.,* 2006). In RSA the incidence of MCC deficiency is not known and no molecular work has been done before the onset of this study. Therefore the implementation of the molecular characterisation of MCC deficiency in SA was needed. Two complementing technical approaches were initiated. The first was the characterisation of the two MCC ORFs as a whole from mRNA using RT-PCR. The second was the genomic characterisation of *mccA* and *mccB* were studied focussing on selected exons that are associated with Caucasian individuals (Chapter 4). This includes exons *mccA*8 and *mccA*11 of the α-subunit and *mccB*5 and *mccB*6 of the β-subunit (Baumgartner *et al.,* 2001; Gallardo *et al.,* 2001; Holzinger *et al.,* 2001; Dantas *et al.,* 2005). A summary of all the described mutations associated with MCC deficiency is listed in Tables 2.2 and 2.3.

Both the RT-PCR, using mRNA (total RNA) as starting point, and the exon-specific PCR where gDNA was used as template, was implemented successfully. Unexpected results were obtained. Cultured fibroblast cells of a MCC non-deficient individual, CFC, were used to implement the molecular characterisation of MCC deficiency to be able to characterize MCC deficiency. Urine metabolic profiles of the individual, CFC, showed no elevated levels of MCC deficiency associated metabolites and was used as a control regarding MCC deficiency. Although another IEM i.e. gangliosidosis I, was suspected. As CFC was a negative control individual for MCC deficiency no

mutations and polymorphisms were expected in the MCC genes (*mccA* and *mccB*). Good quality DNA and RNA was isolated from CFC cultured fibroblasts (Chapters 3 and 4). cDNA syntheses and amplification of the entire ORFs of *mccA* and *mccB* as well as PCR amplification of selected exons on a genomic level was done successfully. Sequence results obtained (chapter 3), revealed that individual CFC has a non-synonymous polymorphism in position 1391A→C of the ORF of the alpha subunit. This single nucleotide change causes an amino acid change of H464P. In the 3'UTR of *mccA* +131 bases from the stop codon of the ORF, another single nucleotide change was observed. Although good quality forward sequence covers this region, this mutation could not be confirmed due to the lack of a good quality reverse strand sequence. The sequencing data of the beta subunit of individual CFC also revealed an unexpected nucleic acid variation. A synonymous SNP, 1368G→A, A456A, recently listed in GenBank (17 November 2006) but not yet described in the literature, is associated with MCC deficient individuals.

The methods that were implemented were used to characterize MCC of a MCC non-deficient individual were subsequently used to characterize MCC of HGS, a South African suffering from MCC deficiency. The diagnosis was based on biochemical analyses and metabolic profiles which indicated elevated levels of the MCC deficiency associated metabolites 3-methylcrotonylglycine, OH-isovalerylcarnitine. Unfortunatly, the MCC activity analysis of this patient has not been done yet. Fibroblst cells of HGS were cultured form a skin biopsy. The cell cultures were established and propagated. Good quality gDNA and RNA ware isolated from the HGS fibroblast cell cultures. Patient HGS had no mutations in the four exons (*mccA*8, *mccA*11, mcB5, *mccB*6) as well as the genomic region *mccB*5-6 that were selected for amplification and sequencing based on their reported association wit MCC deficiency in Caucasians. The entire ORFs of *mccA* and *mccB* of HGS were also amplified. Difficulty in obtaining single amplicons for both *mccA* and *mccB* was experienced under the exact same protocol that was succesfull for RT-PCR of CFC nucleic acids. Although full-length amplicons of the correct size were obtained, there were always several smaller amplicons as well. It is, therefore, recommended that these amplicons should also be sequenced in future experiments. These sequences will indicate whether the amplified products are specific or non-specific. New primers should also be designed to try to improve the specificity of the amplification. The entire ORF of *mccA* of HGS was sequenced. Controversy regarding position 1391 of the alpha subunit HGS*mccA* arises. A great possibility must of a 1391C→A heterozygous mutation. The lack of non-coding strand sequences resulted in an uncertain identification of the nucleic acid in this 1391 position of the HGS*mccA* amplicons. A shortcoming in the characterisation of *mccB* of patient HGS was the poor sequence data obtained from some of the sequencing primers. The sequences of the primers were obtained from personal communication with Dr. M.R.

Baumgartner. It is not possible at this stage to determine if the problem is fundamental or technical. If the regions chosen for the primers are hotspots for mutations a new region should be identified. If poor quality primers were synthesised, a new batch must be considered. After several attempts in order to determine the sequence of the HGS*mccB* the entire ORF could not be sequenced. This sequence is therefore incomplete and needs to be completed. The region that was not sequenced includes exons 7 and 8.

Another technical drawback with the sequence data presented in this study is that the design of the sequencing primer sets for the coding and non-coding strands was such that even though the complete sequence could be obtained from overlapping regions, the two strands were not fully sequenced. New sequence primers must be designed so that overlapping of the sequence data from the forward primers with each other occurs as well as overlapping of the sequence data from the set of reverse primers. Therefore, to summarize the findings with regard to the MCC deficient patient HGS: He does not have any novel mutation in the exons 1-19 of the alpha subunit, but could be heterozygous for the SNP 1391 A→C in the *mccA* subunit. No mutations were detected in exons 1-6 and 9-17 of *mccB*.

To conclude, the molecular characterisation of MCC deficiency in a South African patient was successful for the amplification and sequencing of the genomic regions. The amplification of HGS*mccA* and HGS*mccB* resulted in multiple fragments. Gel extracted amplicons were sequenced. The amplicons HGS*mccA* was successfully sequenced but a part of the HGS*mccB* could not be sequenced. Therefore, the characterisation of the HGS*mccB* gene is incomplete and needs to be done. At this stage, it is not possible to determine the cause of the clinical presentation of MCC deficient patient (HGS). It is possible for the disease causing mutation to be in the exon 7 and exon 8 regions which was not completely sequenced or in the non-coding regions (introns) of the genes. Extended studies should be launched in order to determine the disease causing characteristic of HGS.

New approaches towards the investigation of molecular aspecs of diseases are summarised here to highlights the possibilities for future studies. As mentioned repeatedly, several groups are investigating MCC deficiency on a molecular and genetic level (Baumgartner *et al.,* 2001; Holzinger *et al.,* 2001; Gallardo *et al.,* 2001; Dantas *et al.,* 2005). To date, it has not been possible to correlate the genotype to the phenotype of individuals. The lack of the genotype-phenotype correlation could be due to the complexity of the disease. In complex diseases, a network of metabolite fluxes might be subtly altered to cause a phenotype (Lanpher *et al.,* 2006).

The pathogenesis of an IEM can generally by attributed to the loss or gain of function of mutant proteins (usually an enzyme or a transporter). The genetic basis of IEMs is extremely heterogeneous and can involve any type of genetic defect: one or more point mutations, deletions or insertions of genomic rearrangements. Mutations can occur in coding or regulatory sequences and mutations in different genes can phenocopy each other by affecting the same pathway. The disease is generally associated with altered metabolic flux through the pathway that is regulated by the mutant protein (Lanpher *et al.,* 2006). Lack of knowledge regarding the understanding of transcriptomic regulation contributes to the difficulties experienced in making genotype-phenotype correlation. The variation in the efficacy of detoxification systems of individuals could also contribute to their phenotypes. For most IEMs, including MCC deficiency, genotype-phenotype correlation is clearly not simple. A more global approach is needed to identify the complexity of factors that determine the clinical outcome of MCC deficiency and other IEMs. It is possible that an individual's genomic variants affect metabolic flux in a subtle fashion, without evidence of clinical disease. The cumulative effects of multiple sequence variations on a network of metabolite fluxes might, however, be sufficient to surpass the clinical threshold for disease, either alone of in combination with environmental factors (Lanpher *et al.,* 2006). So, it is also necessary to understand the effects of genomic changes on the concentrations of the RNA molecules (the transcriptome), relative concentration of the proteins (the proteome), the nature of protein-protein interactions, that is the interactome as well as the concentration of the metabolites that exist in any metabolic environmental state, (the metabolome).

Transcriptome analyses or global gene expression profiling has become a valuable tool in research. Recent developments have shown that the transcriptome is considerably larger and more divergently expressed than was previously thought (Carter, 2006). Small physiologically functional RNAs are now known to be nested within the large intronic regions of primary RNA transcripts (Carter, 2006). It is commonly accepted that different cell types exhibit distinct patterns of gene expression that reflect specific physiological functions. However, the full reality of cellular transcriptome variance is only now being revealed by cellular transcriptomic studies. Gene expression is also highly dependent upon physiological state; recent studies have shown that this also applies non-coding RNAs, including antisense transcripts (Carter, 2006).

These new developments are all applicable to study MCC and other IEMs with a more global and systemic approach rather than a specific more rigid approach.

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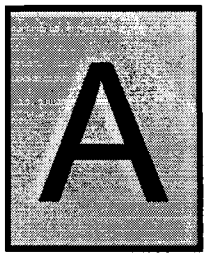
APPENDIX



Sequences of *mccA*

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 121 ggctccaggg acatggcggc ggcctctgcg gtgtcggtgc tgctggtggc ggcggagagg
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 421 tccatcggcc ccgctccctc ccagcagagc tacctatcta tggagaaaat cattcaagtg
 481 gccaagacct ctgctgcaca ggctatccat ccaggatgcg gttttctttc agaaaacatg
 541 gaatttgctg aactttgtaa gcaagaagga attatttta taggccctcc tccatctgca
 601 attagagaca tgggtataaa gagcacatcc aaatccataa tggctgctgc tggagtacct
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1441 ctggtcgtgt gggcagcaga tcgccaggcg gcattgacaa aactgaggta cagccttcgt
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1681 ctcaaggaga aagccatgac cgacactttc actcttcagg cacatgatca attctctcca
1741 ttttcgtcta gcagtggaag aagactgaat atctcgtata ccagaaacat gactcttaaa
1801 gatggtaaaa acaatgtagc catagctgta acgtataacc atgatgggtc ttatagcatg
1861 cagattgaag ataaaacttt ccaagtcctt ggtaatcttt acagcgaggg agactgcact
1921 tacctgaaat gttctgttaa tggagttgct agtaaagcga agctgattat cctggaaaac
1981 actatttacc tattttccaa ggaaggaagt attgagattg acattccagt ccccaaatac
2041 ttatcttctg tgagctcaca agaaactcag ggcggcccct tagctcctat gactggaacc
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```

Figure A1: Transcript sequence *mccA* (AF 310339 GenBank).....CONTINUE

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2161 atgatcgcca tgaagatgga gcataccata aagtctccaa aggatggcac agtaaagaaa
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2401 agcagtttta ctaaatgatt gtatgcttat gctgaacccc tttcatattg gagaatcatg
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2521 tggaagcc
```

Figure A1: Transcript sequence *mccA* (AF 310339 GenBank)

```
M A A A S A V S V L L V A A E R N R W H R L P S L L L P P R T W V W R Q R
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T V A V Y S E A D R N S M H V D M A D E A Y S I G P A P S Q Q S Y L S M E
K I I Q V A K T S A A Q A I H P G C G F L S E N M E F A E L C K Q E G I I
F I G P P P S A I R D M G I K S T S K S I M A A A G V P V V E G Y H G E D
Q S D Q C L K E H A R R I G Y P V M I K A V R G G G G K G M R I V R S E Q
E F Q E Q L E S A R R E A K K S F N D D A M L I E K F V D T P R H V E V V
F G D H H G N A V Y L F E R D C S V Q R R H Q K I I E E A P A P G I K S E
V R K K L G E A A V R A A K A V N Y V G A G T V E F I M D S K H N F C F M
E M N T R L Q V E H P V T E M I T G T D L V E W Q L R I A A G E K I P L S
Q E E I T L Q G H A F E A R I Y A E D P S N N F M V A G P L V H L S T P R
A D P S T R I E T G V R Q G D E V S V H Y D P M I A K L V V W A A D R Q A
A L T K L R Y S L R Q Y N I V G L P T N I D F L L N L S G H P E F E A G N
V H T D F I P Q H H K Q L L L S R K A A A K E S L C Q A A L G L I L K E K
A M T D T F T L Q A H D Q F S P F S S S S G R R L N I S Y T R N M T L K D
G K N N V A I A V T Y N H D G S Y S M Q I E D K T F Q V L G N L Y S E G D
C T Y L K C S V N G V A S K A K L I I L E N T I Y L F S K E G S I E I D I
P V P K Y L S S V S S Q E T Q G G P L A P M T G T I E K V F V K A G D K V
K A G D S L M V M I A M K M E H T I K S P K D G T V K K V F Y R E G A Q A
N R H T P L V E F E E E E S D K R E S E
```

Figure A2: Translation sequence *MCCA* (AF 310339 GenBank)

Exon sequences extracted from genomic DNA sequence ENST00000265594

....**CCTAACAGTTTAGCTTAACG**AATGTGATATTTATCACCCCTAACAAATATATTAACATACATGTTTGCT
TTTATATAG**GCATGTAGAAGTCCAGGTGTTTGGTGATCACCATGGCAATGCTGTGTACTTGTTTGAAAGAGAC**
TGTAGTGTGCAGAGGCGACATCAGAAGATCATTGAGGAGGCCCCAGCGGTAAGGACCTTGGAAAGAAATTTGT
AAGCTTCTC**CGTAATGTGAATCTTACTCTG**....

Figure A3: Sequence of *mccA* 8; the highlighted sequence indicate *mccA* exon 8. The underlined sequences indicate the locations of intron-specific primers (Sequences adapted from Ensembl, 2005).

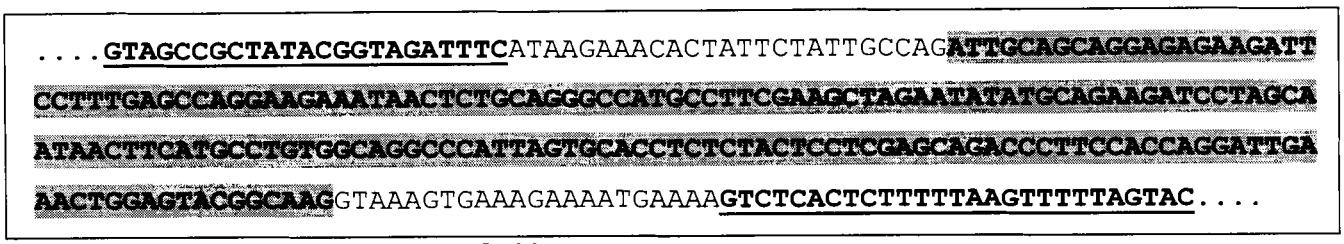

Figure A4: Sequence of *mccA* 11; the highlighted sequence indicate *mccA* exon 11. The underlined sequences indicate the locations intron-specific primers (Sequences adapted from Ensembl, 2005).

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                                1                                                                                                   100
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       MCCA1-19_1          (1) GCCCAAAGGTAGGCTCAGGCTCCGACGGTGGCCGGCGGGGGTCACGAGGCTTCGTAGTGGAGGAACGGGTTTGGCGTGTGGGACGCAGCTGCCTCTGTAC
       MCCA1-19_2          (1) ----------------------------------------------------------------------------------------------------
       MCCA1-19_3          (1) ----------------------------------------------------------------------------------------------------
       MCCA1-19_4          (1) ----------------------------------------------------------------------------------------------------
       MCCA1-19_5          (1) ----------------------------------------------------------------------------------------------------
       MCCA1-19_6          (1) ----------------------------------------------------------------------------------------------------
       MCCA1-19_7          (1) ----------------------------------------------------------------------------------------------------
       MCCA1-19_8          (1) ----------------------------------------------------------------------------------------------------
   CFC_MCCA1-19_8          (1) ----------------------------------------------------------------------------------------------------
    LZ_MCCA1-19_8          (1) ----------------------------------------------------------------------------------------------------
   CVH_MCCA1-19_8          (1) ----------------------------------------------------------------------------------------------------
       MCCA1-19_9          (1) ----------------------------------------------------------------------------------------------------
      MCCA1-19_10          (1) ----------------------------------------------------------------------------------------------------
      MCCA1-19_11          (1) ----------------------------------------------------------------------------------------------------
  CFC_MCCA1-19_11          (1) ----------------------------------------------------------------------------------------------------
  CVH_MCCA1-19_11          (1) ----------------------------------------------------------------------------------------------------
   LZ_MCCA1-19_11          (1) ----------------------------------------------------------------------------------------------------
      MCCA1-19_12          (1) ----------------------------------------------------------------------------------------------------
      MCCA1-19_13          (1) ----------------------------------------------------------------------------------------------------
      MCCA1-19_14          (1) ----------------------------------------------------------------------------------------------------
      MCCA1-19_15          (1) ----------------------------------------------------------------------------------------------------
      MCCA1-19_16          (1) ----------------------------------------------------------------------------------------------------
      MCCA1-19_17          (1) ----------------------------------------------------------------------------------------------------
      MCCA1-19_18          (1) ----------------------------------------------------------------------------------------------------
      MCCA1-19_19          (1) ----------------------------------------------------------------------------------------------------

                              101                                                                                                   200
AF310339 MCCA1-19        (100) TGGGGAGTCACGGAGTGGCCGGGCTCCAGGGACATGGCGGCGGCCTCTGCGGTGTCGGTGCTGCTGGTGGCGGCGGAGAGGAACCGGTGGCATCGTCTCC
AF310972 MCCA1-19        (101) TGGGGAGTCACGGAGTGGCCGGGCTCCAGGGACATGGCGGCGGCCTCTGCGGTGTCGGTGCTGCTGGTGGCGGCGGAGAGGAACCGGTGGCATCGTCTCC
       MCCA1-19_1        (101) TGGGGAGTCACGGAGTGGCCGGGCTCCAGGGACATGGCGGCGGCCTCTGCGGTGTCGGTGCTGCTGGTGGCGGCGGAGAGGAACCGGTGGCATCGTCTCC
       MCCA1-19_2          (1) ----------------------------------------------------------------------------------------------------
       MCCA1-19_3          (1) ----------------------------------------------------------------------------------------------------
       MCCA1-19_4          (1) ----------------------------------------------------------------------------------------------------
       MCCA1-19_5          (1) ----------------------------------------------------------------------------------------------------
       MCCA1-19_6          (1) ----------------------------------------------------------------------------------------------------
       MCCA1-19_7          (1) ----------------------------------------------------------------------------------------------------
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   CVH_MCCA1-19_8          (1) ----------------------------------------------------------------------------------------------------
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      MCCA1-19_10          (1) ----------------------------------------------------------------------------------------------------
      MCCA1-19_11          (1) ----------------------------------------------------------------------------------------------------
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  CVH_MCCA1-19_11          (1) ----------------------------------------------------------------------------------------------------
   LZ_MCCA1-19_11          (1) ----------------------------------------------------------------------------------------------------
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Figure A5: Alignment of mature mRNA transcripts with exons 1-19 of *mccA* and exons *mccA*8 and *mccA*11 amplified from individuals CFC, CVH and LZ

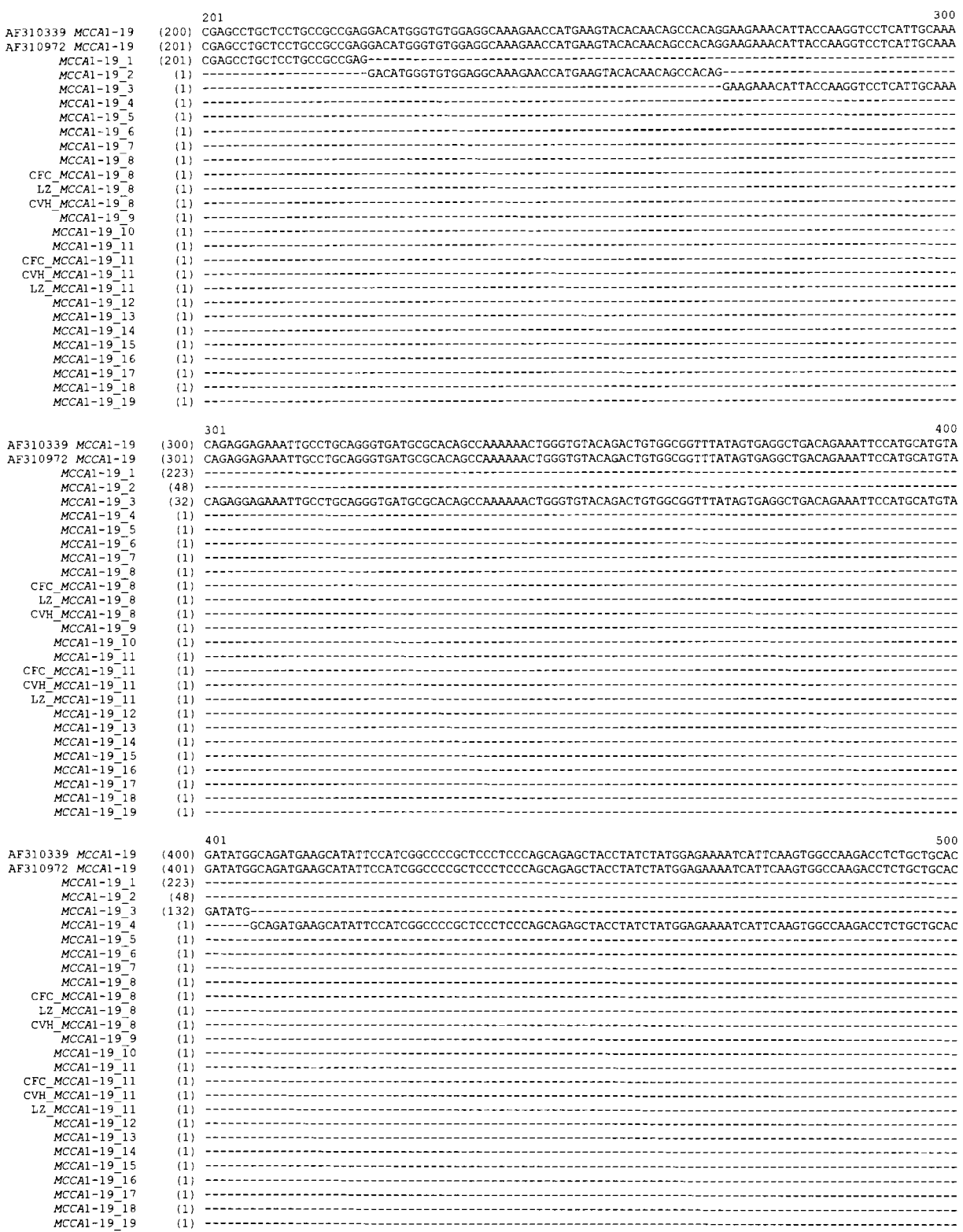


Figure A5: Alignment of mature mRNA transcripts with exons 1-19 of *mccA* and exons *mccA*8 and *mccA*11 amplified from individuals CFC, CVH and LZ .....Continue

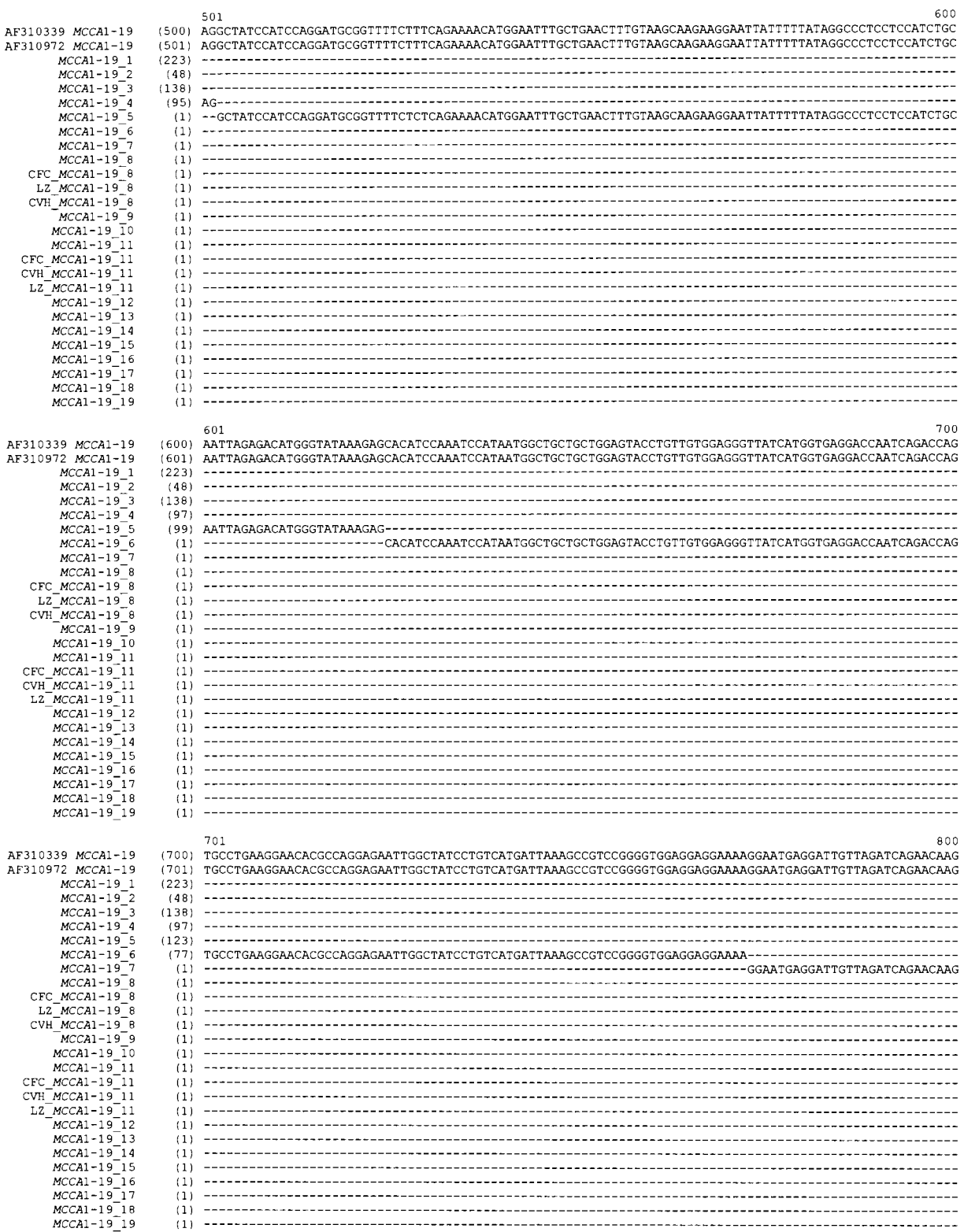


Figure A5: Alignment of mature mRNA transcripts with exons 1-19 of *mccA* and exons *mccA*8 and *mccA*11 amplified from individuals CFC, CVH and LZ .....Continue

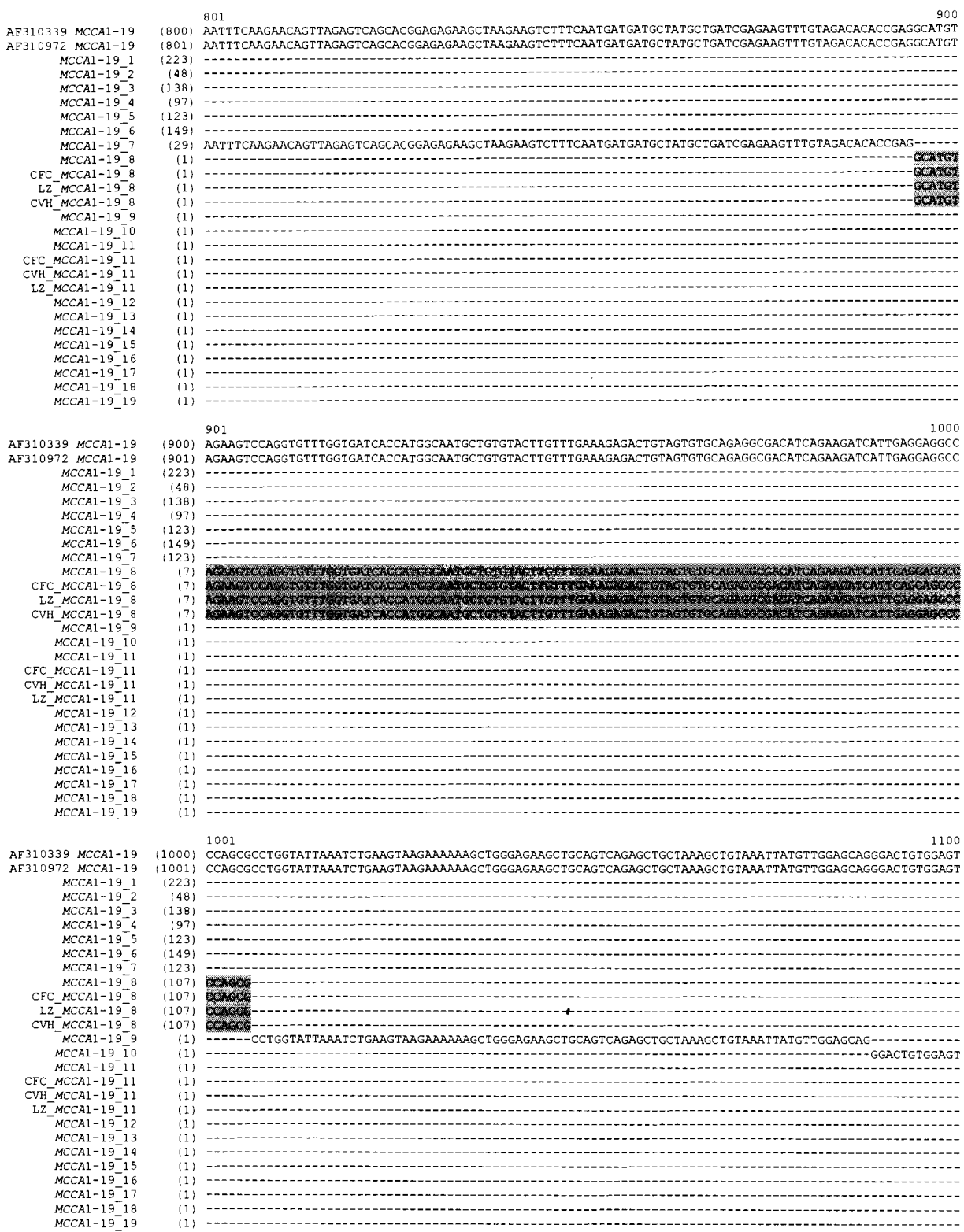


Figure A5: Alignment of mature mRNA transcripts with exons 1-19 of *mccA* and exons *mccA*8 and *mccA*11 amplified from individuals CFC, CVH and LZ .....Continue

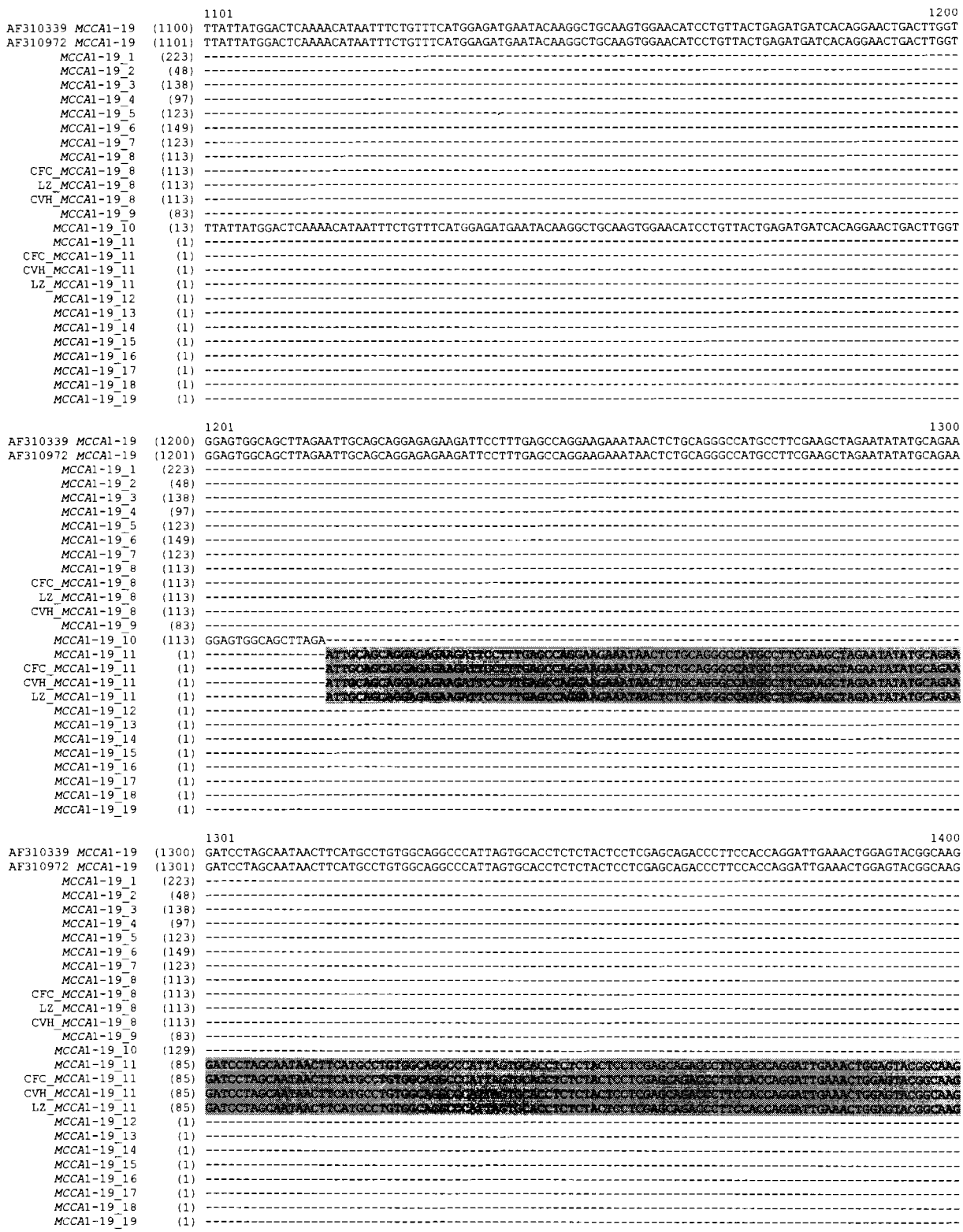


Figure A5: Alignment of mature mRNA transcripts with exons 1-19 of *mccA* and exons *mccA*8 and *mccA*11 amplified from individuals CFC, CVH and LZ .....Continue

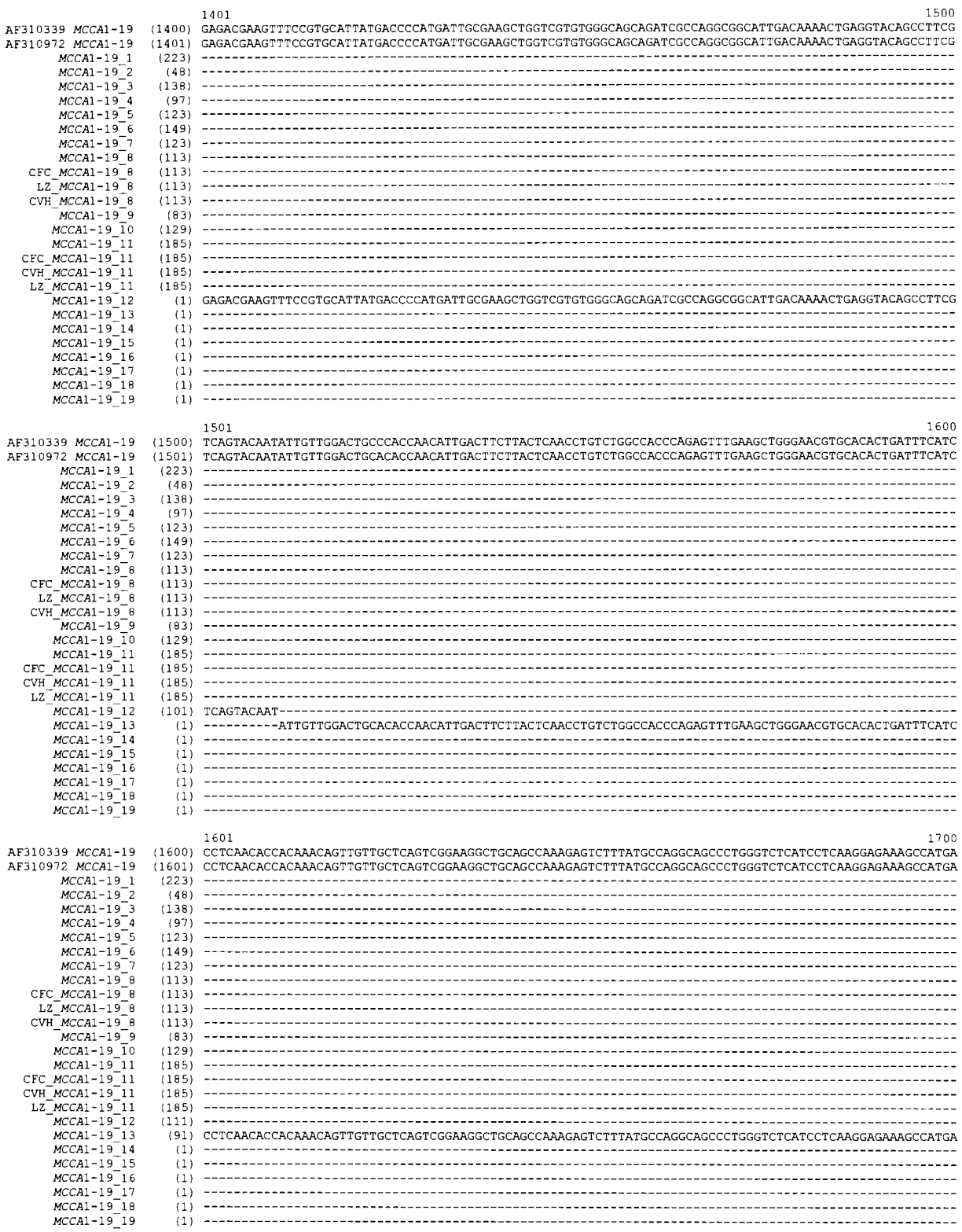


Figure A5: Alignment of mature mRNA transcripts with exons 1-19 of *mccA* and exons *mccA*8 and *mccA*11 amplified from individuals CFC, CVH and LZ .....Continue

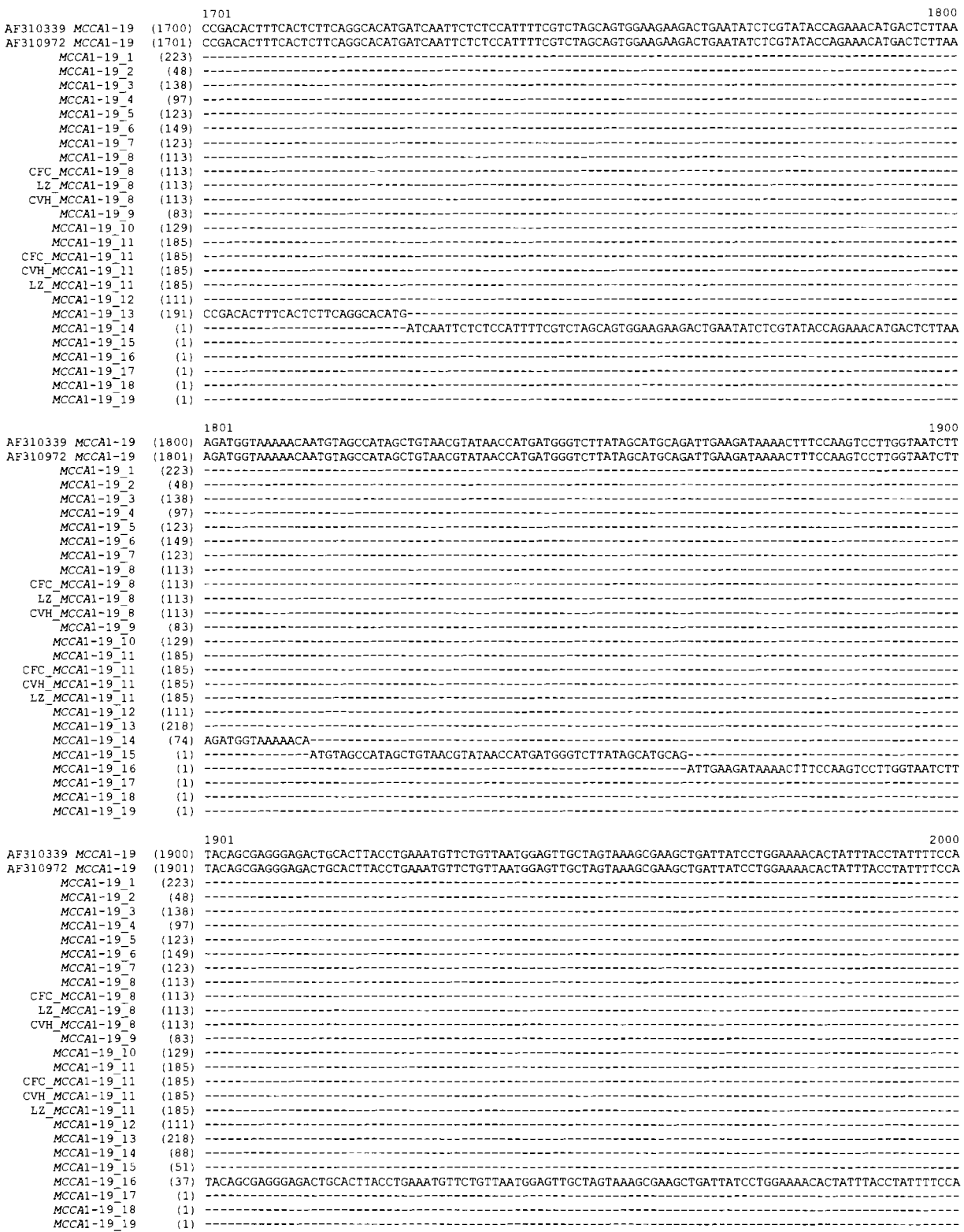
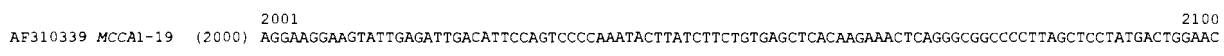


Figure A5: Alignment of mature mRNA transcripts with exons 1-19 of *mccA* and exons *mccA*8 and *mccA*11 amplified from individuals CFC, CVH and LZ .....Continue



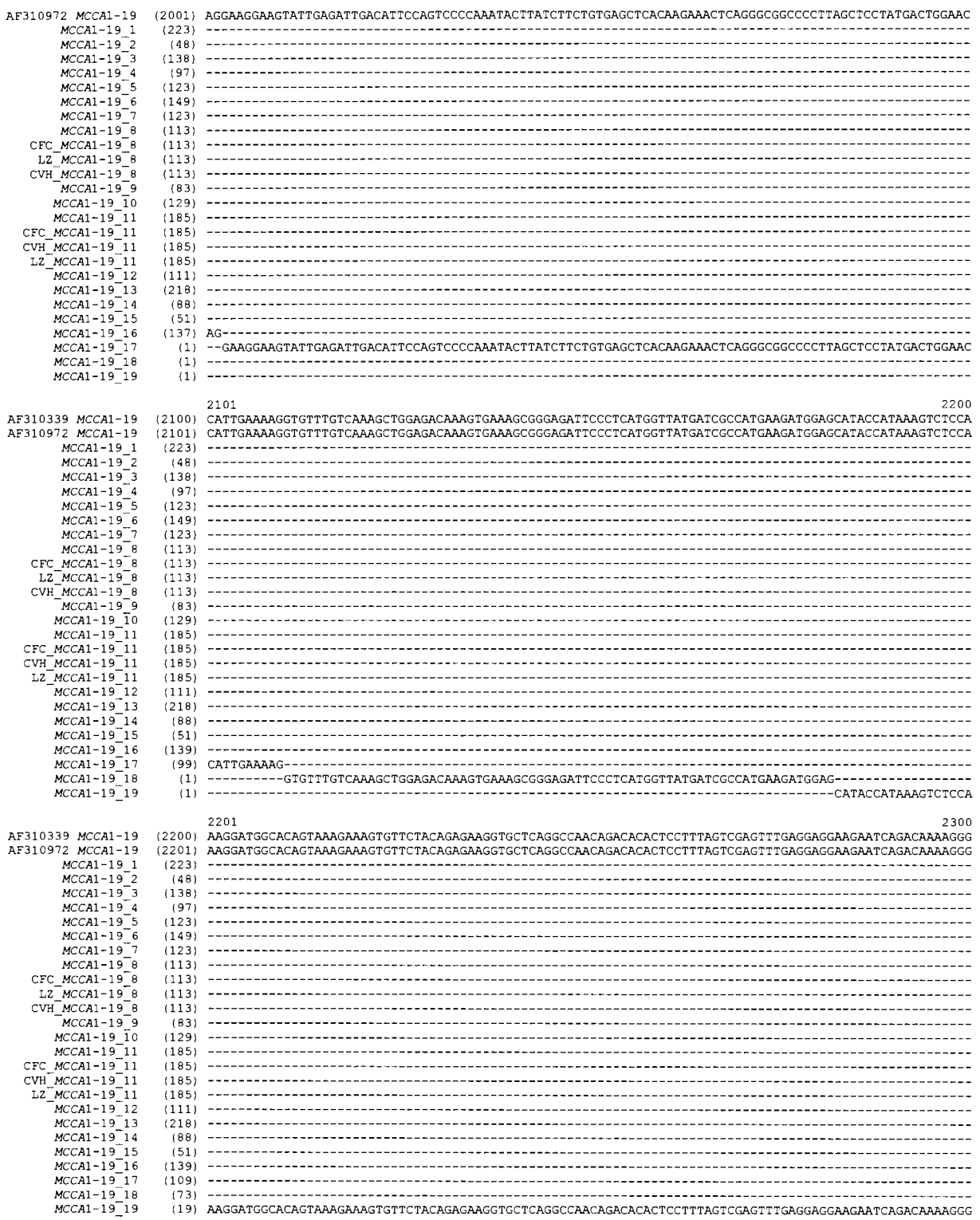


Figure A5: Alignment of mature mRNA transcripts with exons 1-19 of *mccA* and exons *mccA*8 and *mccA*11 amplified from individuals CFC, CVH and LZ .....Continue

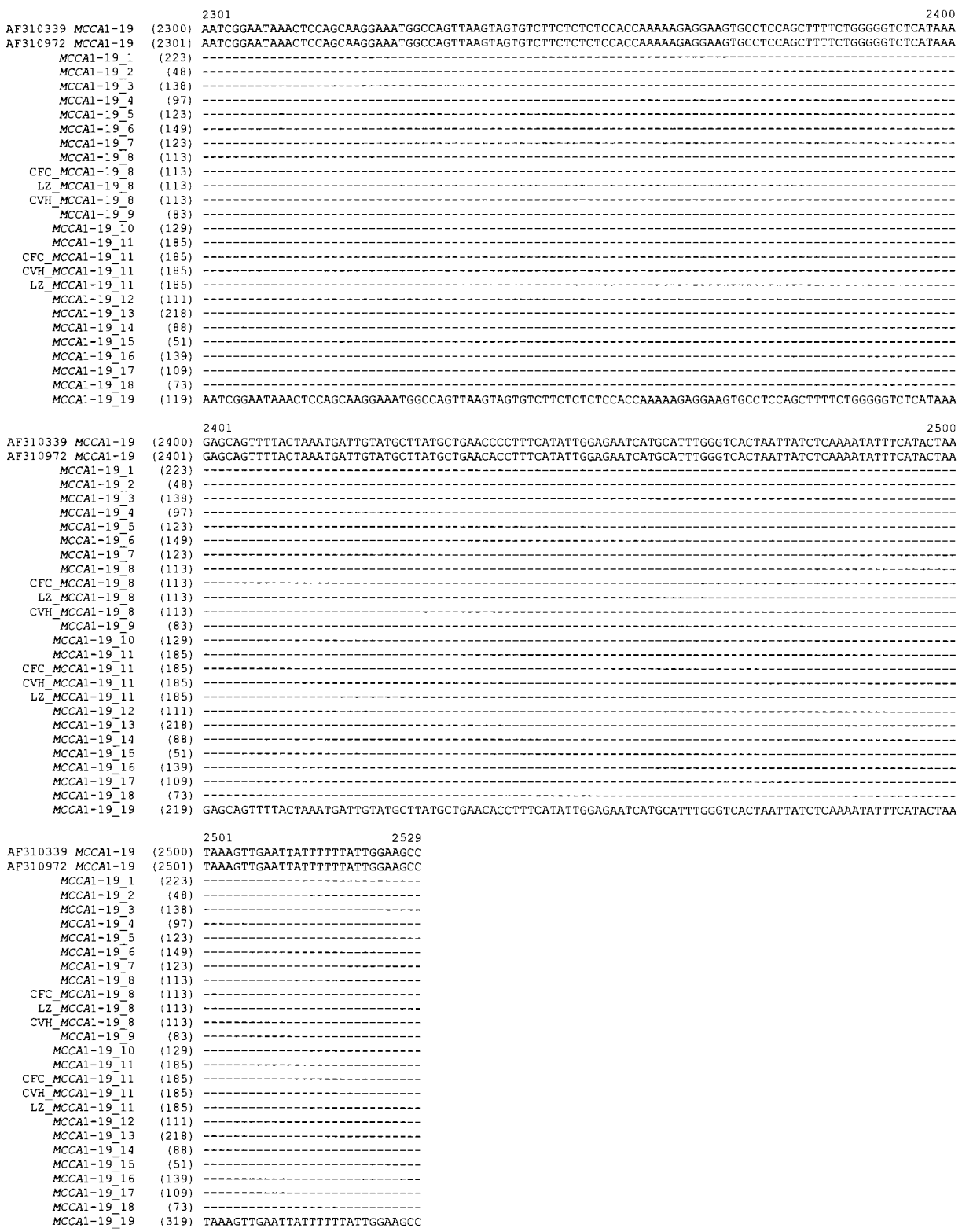
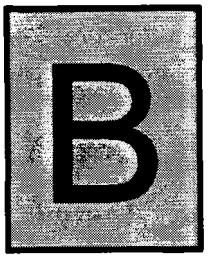


Figure A5: Alignment of mature mRNA transcripts with exons 1-19 of *mccA* and exons *mccA*8 and *mccA*11 amplified from individuals CFC, CVH and LZ

APPENDIX



Sequences of *mccB*

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2041 agcggagaca gtaatttatg gttatccttt ctgacccaca aagtatgaaa agttctgtaa
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2161 actgaaaatg
```

Figure B1: Transcript sequence of *mccB* (AF 310971)

M W A V L R L A L R P C A R A S P A G P R A Y H G D S V A S L G T Q P D L
G S A L Y Q E N Y K Q M K A L V N Q L H E R V E H I K L G G G E K A R A L
H I S R G K L L P R E R I D N L I D P G S P F L E L S Q F A G Y Q L Y D N
E E V P G G G I I T G I G R V S G V E C M I I A N D A T V K G G A Y Y P V
T V K K Q L R A Q E I A M Q N R L P C I Y L V D S G G A Y L P R Q A D V F
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V P A M A D E N I I V R K Q G T I F L A G P P L V K A A T G E E V S A E D
L G G A D L H C R K S G V S D H W A L D D H H A L H L T R K V V R N L N Y
Q K K L D V T I E P S E E P L F P A D E L Y G I V G A N L K R S F D V R E
V I A R I V D G S R F T E F K A F Y G D T L V T G F A R I F G Y P V G I V
G N N G V L F S E S A K K G T H F V Q L C C Q R N I P L L F L Q N I T G F
M V G R E Y E A E G I A K D G A K M V A A V A C A Q V P K I T L I I G G S
Y G A G N Y G M C G R A Y S P R F L Y I W P N A R I S V M G G E Q A A N V
L A T I T K D Q R A R E G K Q F S S A D E A A L K E P I I K K F E E E G N
P Y Y S S A R V W D D G I I D P A D T R L V LG L S F S A A L N A P I E K
T D F G I F R M

Figure B2: Translation sequence of *MCCB* (AF 310971)

Exon sequences extracted from reference genomic DNA sequence ENST00000388991

....**GACTGCTGTCTGCTAATGGATG**TTAATAGTGATACGTACTAGAAGTTGAAGGTTGT**attggggtatctt**
gtaatgagtgTAATTAGTTTTGAAGAAATCTCTTAAATTCTCTCTCCAATGAAATTTCTGCCTTTCAG**AGTAG**
AATGCATGATTATTGCCAATGATGCCACCGTCAAAGGAGGTGCCTACTACCCAGTGACTGTGAAAAAACAATT
ACGGGCCCAAGAAATTGCCATGCAAAACAGGCTCCCCTGCATCTACTTAGGCAAGTCACCAGAGTGGTAAAAT
AAACTATTATTAGCTGGTAAAATGCAA**gatagtttggaaggctgtatgtat**TACATTTGGGATGGGTATTTTA
TAAACCTGTTGATTTCTCCTGTAATTATAAAGGAAACACAGGTT**CTTTGCTGAAAACTCTGGGG**....

Figure B3: Sequence of *MCCB* 5 (425 bp); the highlighted sequence indicate *MCCB* exon 5. The underlined sequences indicate the locations intron-specific primers (Sequences adapted from Ensembl, 2005); uppercase underlined sequences are those used for amplification.

....**TAACAGTTTAGAAAGACAGGGC**AAGTTTTTTGTGAATGTGATTAAGAACTGATGCTGTCATTGATACAA
GTTTCCCTCTGAAGTTTTTTGTGAATGTGATTAAGAACTGATGCTGTCATTGATACAAGTTTCCCTCTG**cgta**
gcacatttagttcatagagATGCTTATGTTTCTCATTTCTTGTCTTCAGTT**GATTCGGGAGGAGCATACTTAC**
CTCGACAAGCAGATGTGTTTCCAGATCGAGACCACTTTGGCCGTACATTCTATAATCAGGCAATTATGTCTTC
TAAAAATATTGCACAGGTAATTTTTCATGAATAAAGTGTACAGTGGTGCTTTTTACTCTTAAGTATCTTTA**CG**
AATTAGGTACTCTGGGATGGCT....

Figure B4: Sequence of *MCCB* 6 (245 bp; 383 bp); the highlighted sequence indicate *MCCB* exon 6. The underlined sequences indicate the locations intron-specific primers (Sequences adapted from Ensembl, 2005); uppercase underlined sequences are those used for amplification.

....**GACTGCTGTCTGCTAATGGATG**TTAATAGTGATACGTACTAGAAGTTGAAGGTTGT**attggggtatctt**

gtaatgagtgTAATTAGTTTTGAAGAAATCTCTTAAATTCTCTCTCCAATGAAATTTCTGCCTTTCAG**AGTAG**

AATGCATGATTATTGCCAATGATGCCACCGTCAAAGGAGGTGCCTACTACCCAGTGACTGTGAAAAAACAATT

ACGGGCCCAAGAAATTGCCATGCAAAACAGGCTCCCCTGCATCTACTTAGGCAAGTCACCAGAGTGGTAAAAT

AAACTATTATTAGCTGGTAAAATGCAAGATAGTTTGGAAGGCTGTATGTATTACATTTGGGATGGGTATTTTA

TAAACCTGTTGATTTCTCCTGTAATTATAAAGGAAACACAGGTTCTTTGCTGAAAACTCTGGGGGAAAGTAAA

CAAGAACTGTTCATAGTACTAGTGCATAATACTCATACATTTTGACGCATGCCAGTTTTTTTCTCTATGTGTT

GTGTGGTTTTTTTTTTTGTTCTCTAGATCCTGTTTTTCTTTTAACTTTATGGATCATACTATGAAAAAAATA

GTAGCAAGTATTTATAGATCTCTTGAGTGCCAGGTACTGAGTTAAATGTGCAAGTATTATATAATTTGAGCAG

ATGTAATTTAAAGTAAATTTAATTTCCAGTGAAAGTAAACTTATATTTGAAATTAAAACACATTTAAATCAAA

GAACCTGAAAGCAAAGCAGTTTAAAATGGGCCTTTCCTCCGGGCGCGGTGGCTCACGCCTGTAATCCCAGCAC

TTTGGGAGGCCGAGGCAGGCGGATCATGAGGTCAGGAGATGGAGACCATCCTGGCTAACACACTGAAACCTCG

TCTCTACTAAAAAAAAATACAAAAAATTAGCCGAGCGTGGTGGCAGGCAGCTGTAGTCCCAGCTACTTGGGAG

GCTGAGGCAGGAGAATGGTGTGAACCTGGGAGGCGGAGCTTGCAGTGAGCTGAAATCGTGCCACTGTACTCCA

GCCTGGGCGACAGAGAGAGACTGTCTCAAAAAAAAAAAAAAAAAAAAAAATGGGCCTTTCCTGATACTGG

GTTAGTCTGATAAAATTCCAACATACGTTGTTCATGTATGCAGGTATTTGAACGGGTACTGATGTAGAGGTAT

TTTTTGTTTTGTGTTTTTCTGTGAAGTTGAATTTACGTGATTAACTCACAATACGAACTGCTCTGCACGTAGA

GAAAACAGCAAATGAGGCCCAGCTGCTTGTAGGTGCTGTGCTGGTCTTTATAGGGGATACAGTGAGGAGGATT

TTGTTGTCTCTGTCTTCAGCGAGCTCATAATGAATTGGACACAGCATATAATGTGCAACCAACAAAAGACTTA

ATGGTTGCAGATTCTGAACTAAGTGCTTTCTATACATTTTTCAAGTCATCCTAATATATGATATGGCTCCTTT

TTTTATCTTCATTTCGTGGGTAAGGAAACCGGGGCATAGGATGGCTAGGAAACACACCTATAATCACACAGCT

GTGAAGTCATGAGCTCTGCTTTGAACGCAACTGACCTGTGTACCCACCACTCCAATCTGCAATGAAAACAGTA

TCAGAAGGCAATATGTATAAATTATTATATGAATTCTGTGGATGGCTTTTGTATTGGTTTAGAAGAAGGAAAG

ATACTTTTCCAGCTTAACTCTTTAAGAATTTATGATGTTTCTGGGGCTTTAGCTGGTCCTCAAAGGAGAGAAG

GTTCTAACCATTTCGAGAAGAATTTTGCACAGAGGTGGGAAAGCCTGATGGGTTTGGGGCAATGAATCCTTTT

GGCTGCATTGGGGGTTTGGAGAACGAGGCTCTAtaacagtttagaaagacagggcAAGTTTTTTGTGAATGTG

ATTAAGAACTGATGCTGTCATTGATACAAGTTTCCCTCTGCGTAGCACATTTAGTTCATAGAGATGCTTATGT

TTCTCATTTCTTGTCTTCAG**TTGATTCGGGAGGAGCATACTTACCTCGACAAGCAGATGTGTTTCCAGATCGA**

GACCACTTTGGCCGTACATTCTATAATCAGGCAATTATGTCTTCTAAAAATATTGCACAGGTAATTTTTCATG

AATAAAGTGTACAGTGGTGCTTTTTACTCTTAAGTATCTTTA**CGAATTAGGTACTCTGGGATGGCT**....

Figure B5: Sequence of *MCCB* 5-6 (2179 bp); the highlighted sequence indicate *MCCB* exon 5-6. The sequences in bold indicate exons 5 and 6, the highlighted sequence between those bold sequences is intron 5. The underlined sequences indicate the locations intron-specific primers (Sequences adapted from Ensembl, 2005); uppercase underlined sequences are those used for amplification.

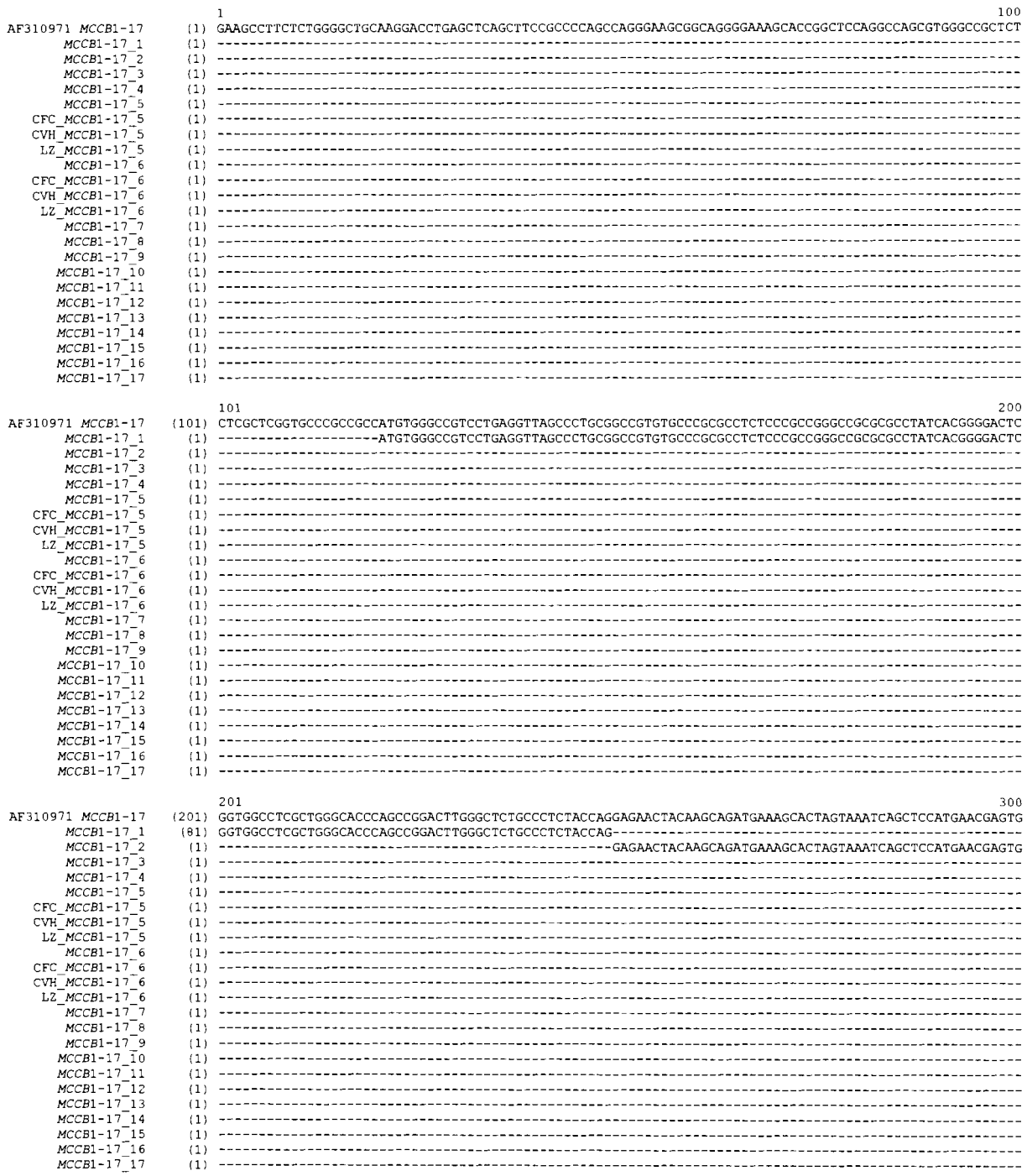


Figure B6: Alignment of mature mRNA transcripts with exons 1-17 of *mccB* and exons *mccB*5 and *mccB*6 amplified from individuals CFC, CVH and LZ.....Continue

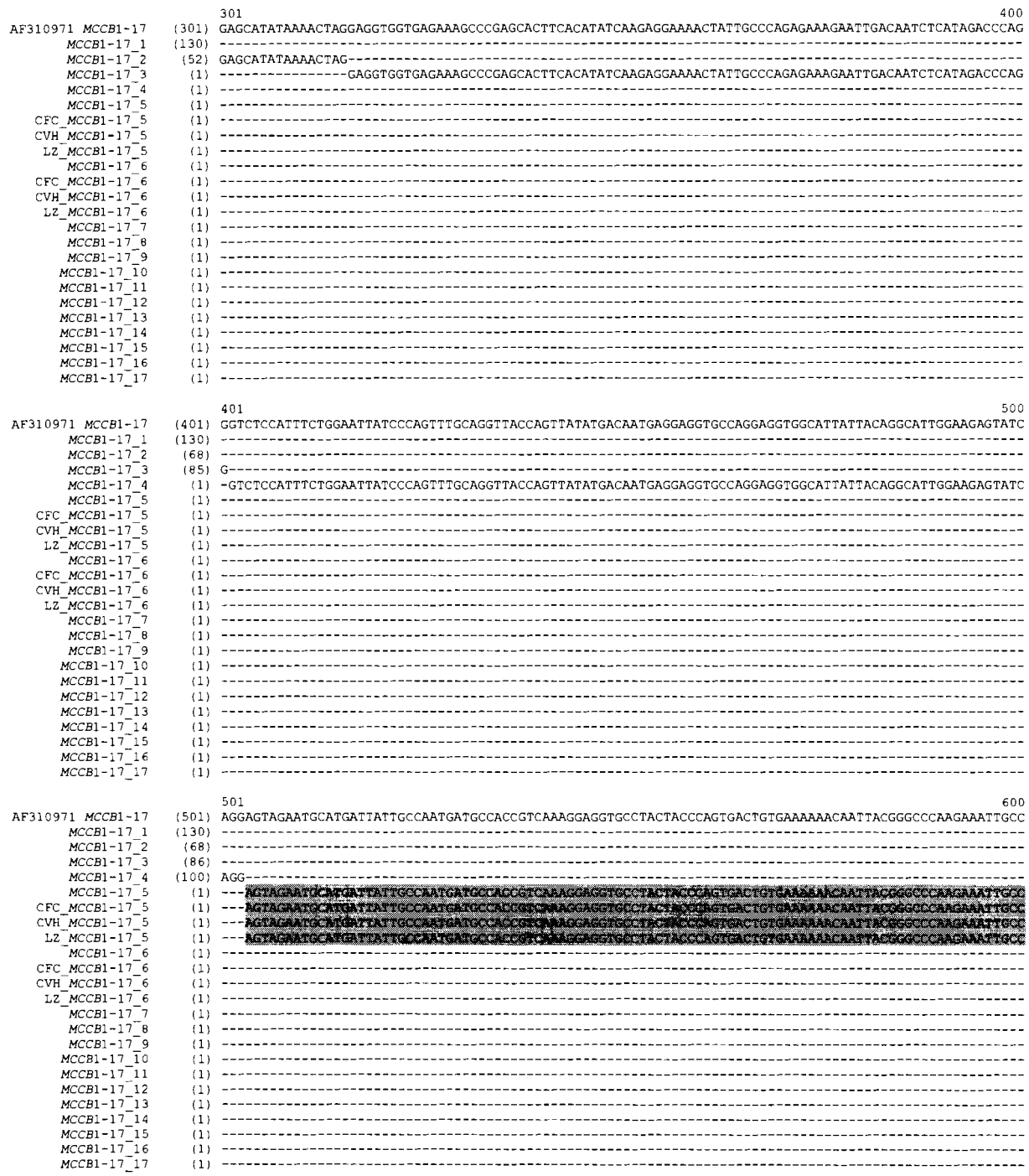


Figure B6: Alignment of mature mRNA transcripts with exons 1-17 of *mccB* and exons *mccB5* and *mccB6* amplified from individuals CFC, CVH and LZ.....Continue

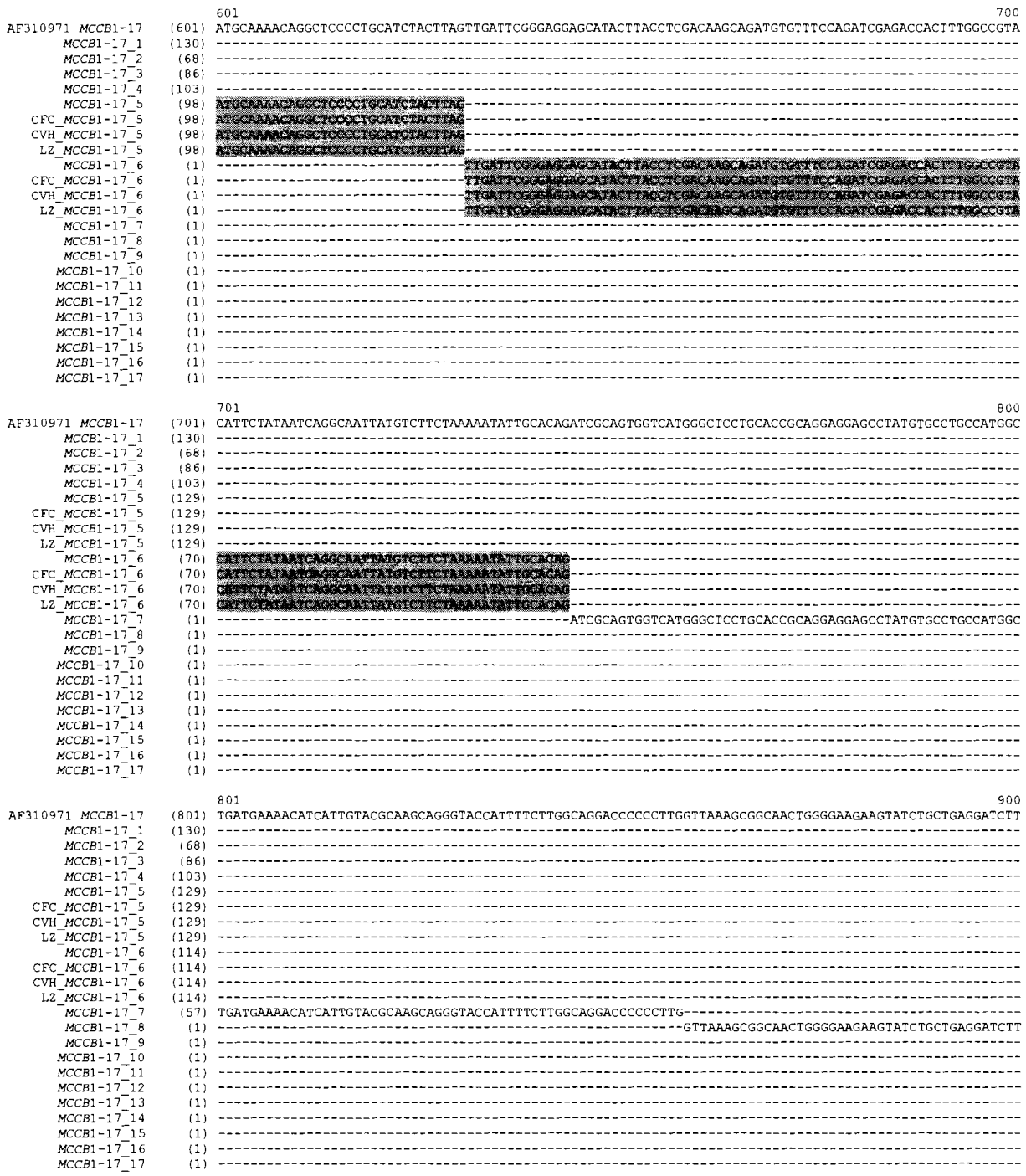


Figure B6: Alignment of mature mRNA transcripts with exons 1-17 of *mccB* and exons *mccB*5 and *mccB*6 amplified from individuals CFC, CVH and LZ.....Continue

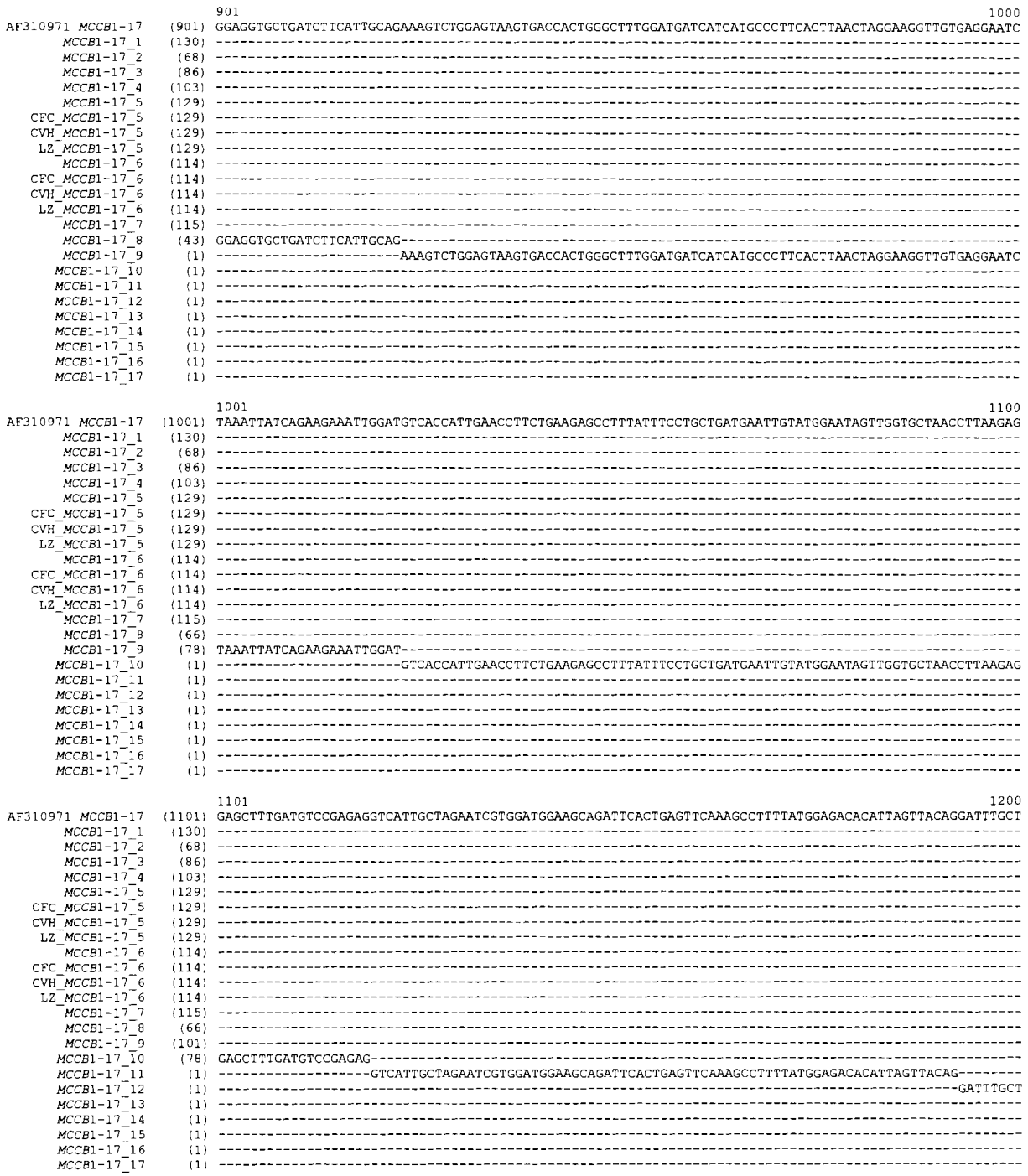


Figure B6: Alignment of mature mRNA transcripts with exons 1-17 of *mccB* and exons *mccB*5 and *mccB*6 amplified from individuals CFC, CVH and LZ.....Continue

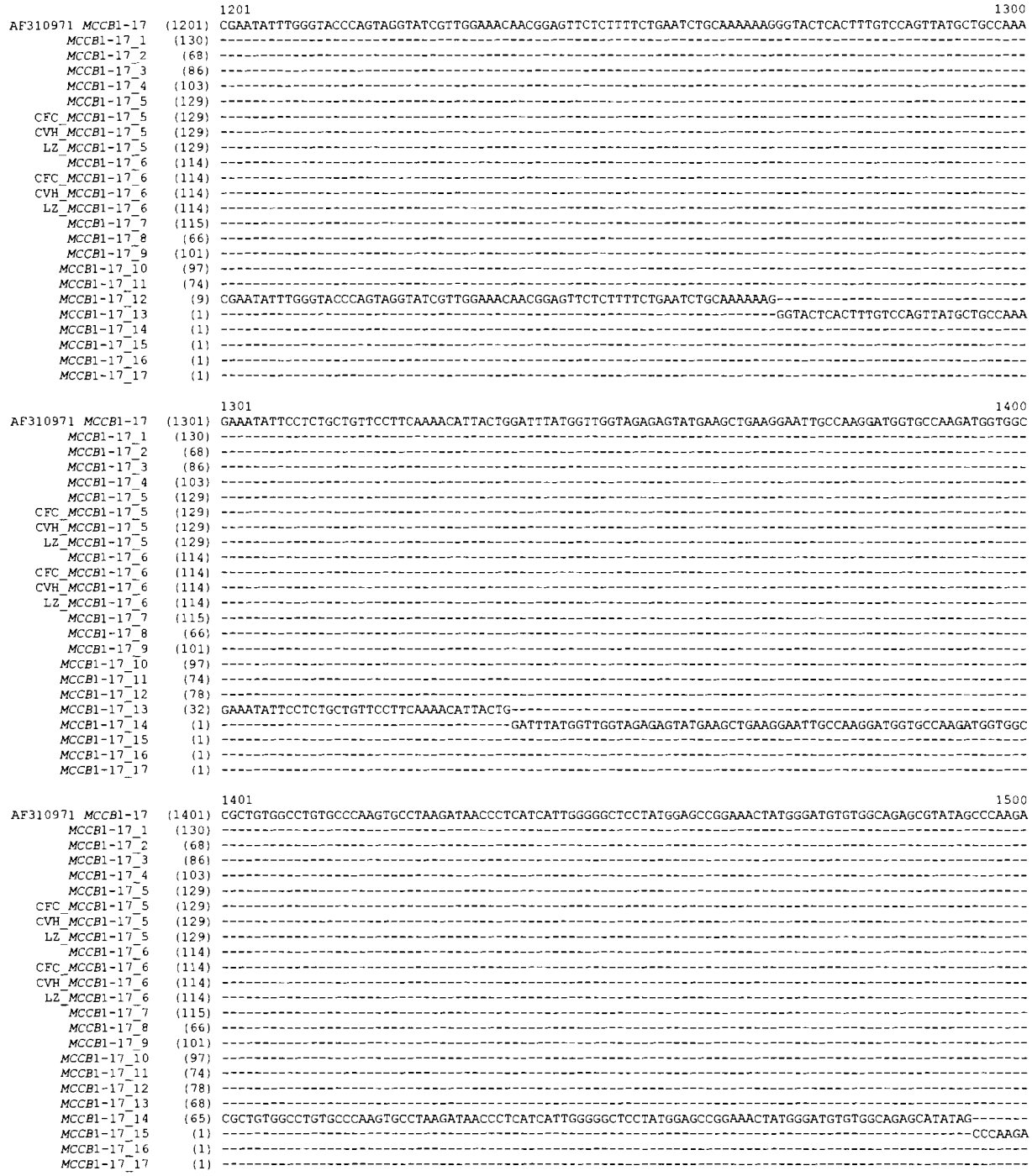


Figure B6: Alignment of mature mRNA transcripts with exons 1-17 of *mccB* and exons *mccB*5 and *mccB*6 amplified from individuals CFC, CVH and LZ.....Continue

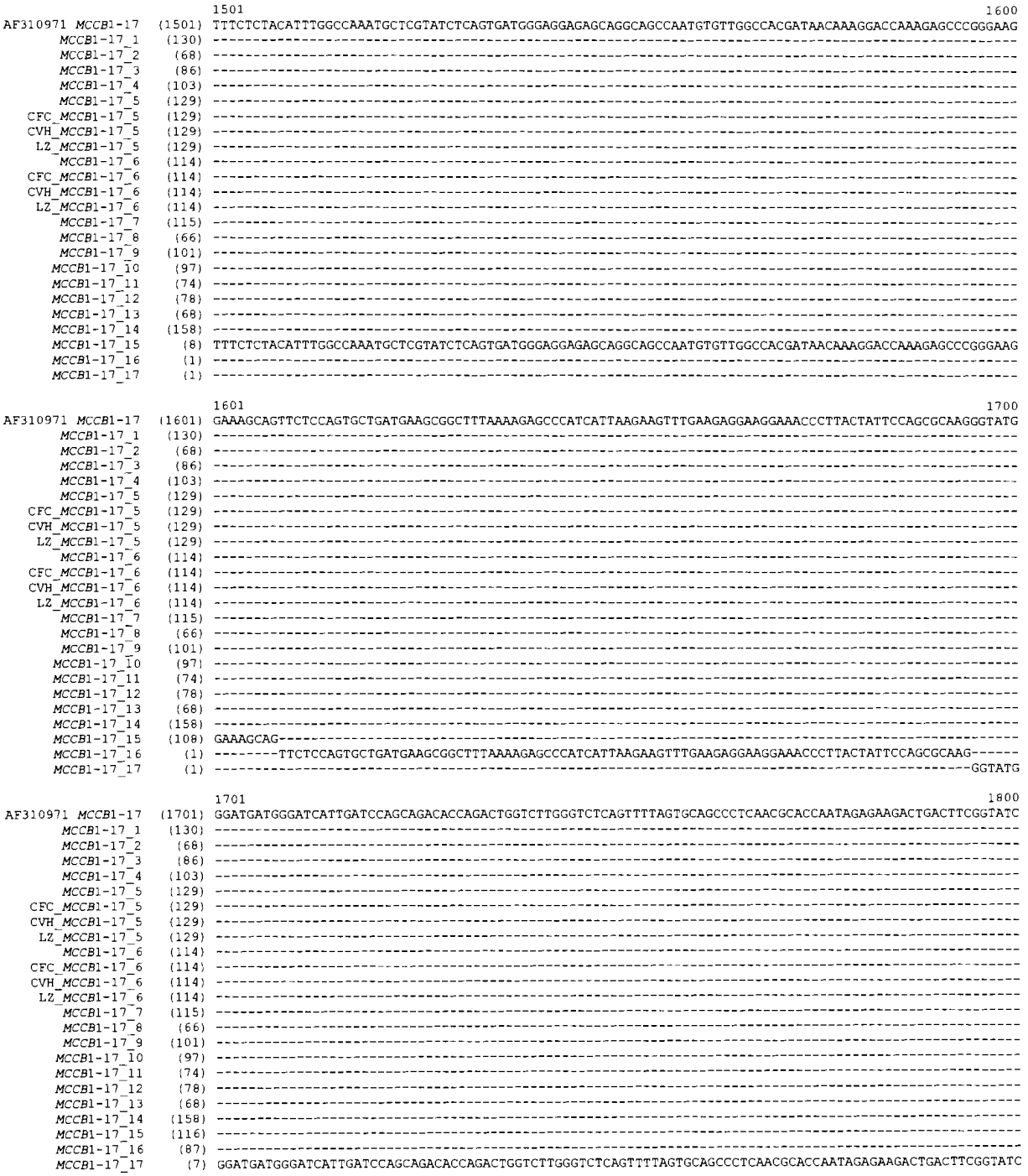


Figure B6: Alignment of mature mRNA transcripts with exons 1-17 of *mccB* and exons *mccB5* and *mccB6* amplified from individuals CFC, CVH and LZ.....Continue

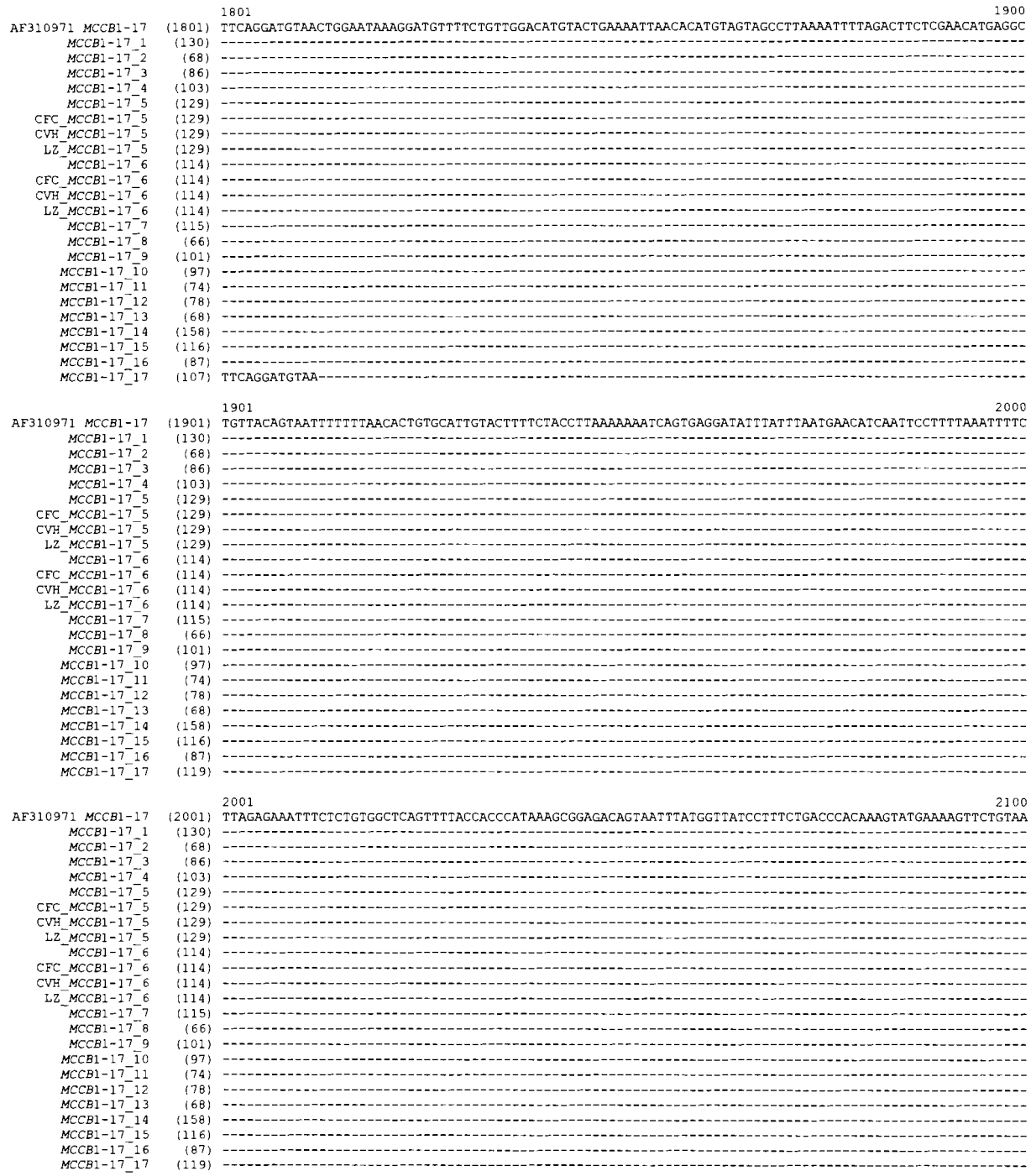
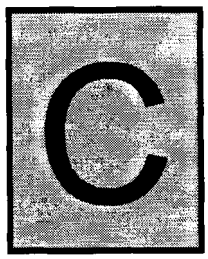


Figure B6: Alignment of mature mRNA transcripts with exons 1-17 of *mccB* and exons *mccB*5 and *mccB*6 amplified from individuals CFC, CVH and LZ.....Continue

Figure B6: Alignment of mature mRNA transcripts with exons 1-17 of *mccB* and exons *mccB*5 and *mccB*6 amplified from individuals CFC, CVH and LZ

APPENDIX

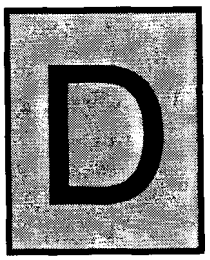
Sequences of *GAPDH*




```
   1 aaattgagcc cgcagcctcc cgcttcgctc tctgctcctc ctgttcgaca gtcagccgca
  61 tcttcttttg cgtcgccagc cgagccacat cgctcagaca ccatgggGAA GGTGAAGGTC
 121 GGAGTCAACG GATTTGGTCG TATTGGGCGC CTGGTCACCA GGGCTGCTTT TAACTCTGGT
 181 AAAGTGGATA TTGTTGCCAT CAATGACCCC TTCATTGACC TCAACTACAT GGTTTACATG
 241 TTCCAATATG ATTCCACCCA TGGCAAATTC CATGGCACCG TCAAGGCTGA GAACGGGAAG
 301 CTTGTCATCA ATGGAAATCC CATCACCATC TTCcaggagc gagatccctc caaaatcaag
 361 tggggcgatg ctggcgctga gtacgtcgtg gagtccactg gcgtcttcac caccatggag
 421 aaggctgggg ctcatttgca gggggggagcc aaaagggtca tcatctctgc cccctctgct
 481 gatgccccca tgttcgtcat gggtgtgaac catgagaagt atgacaacag cctcaagatc
 541 atcagcaatg cctcctgcac caccaactgc ttagcacccc tggccaaggt catccatgac
 601 aactttggta tcgtggaagg actcatgacc acagtccatg ccatcactgc cacccagaag
 661 actgtggatg gcccctccgg gaaactgtgg cgtgatggcc gcggggctct ccagaacatc
 721 atccctgcct ctactggcgc tgccaaggct gtgggcaagg tcatccctga gctgaacggg
 781 aagctcactg gcatggcctt ccgtgtcccc actgccaacg tgtcagtggt ggacctgacc
 841 tgccgtctag aaaaacctgc caaatatgat gacatcaaga aggtggtgaa gcaggcgtcg
 901 gagggccccc tcaagggcat cctgggctac actgagcacc aggtggtctc ctctgacttc
 961 aacagcgaca cccactcctc caccttTGAC gctggggctg gcattgccct caacgaccac
1021 tttgtcaagc tcatttcctg gtatgacaac gaatttggct acagcaacag ggtggtggac
1081 ctcatggccc acatggcctc caaggagtaa gacccctgga ccaccagccc cagcaagagc
1141 acaagaggaa gagagagacc ctcactgctg gggagtccct gccacactca gtcccccacc
1201 acactgaatc tcccctcctc acagttgcca tgtagacccc ttgaagaggg gaggggccta
1261 gggagccgca ccttgtcatg taccatcaat aaagtaccct gtgctcaacc
```

Figure H1: cDNA sequence (NM002046) The Highlighted section is the amplified region using the primers (the underlined regions) listed in Table 3.5 Section 3.1.4.1 .

APPENDIX



Gangliosidosis type 1

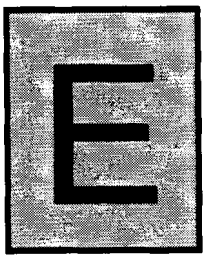
This appendix serves as background regarding the IEM gangliosidosis type 1. The individual CFC, used as MCC non-deficient for the standardisation of methods was suspected to suffer from this disease.

Additional information

This disorder known as Tay-Sachs disease (TSD) is concisely defined by OMIM (Online Mendelian Inheritance in Man) as "an autosomal recessive, progressive neurodegenerative disorder, which in the classic infantile form, is usually fatal by age 2 or 3 years, results from deficiency of the enzyme hexosaminidase A. " "Autosomal" points to the gene for TSD residing on a nonsex (autosomal) chromosome (namely, chromosome15q23-q24). "Recessive" indicates a person with 2 copies of the gene has TSD whereas someone with 1 copy is a carrier in normal health. TSD worsens, with time, as the central nervous system progressively deteriorates. The "classic" ("textbook") type of TSD has its insidious onset in infancy. The child with TSD usually develops normally for the first few months of life. An exaggerated startle reaction may first be noted. Head control is lost by 6-8 months of age. The infant cannot roll over or sit up. Spasticity and rigidity develop. Excessive drooling and convulsions become evident. Blindness and head enlargement set in by the second year. "Fatal by age 2 or 3 years" today would be modified to "fatal by age 5." After age 2, total constant nursing care is needed. Death is due usually to cachexia (wasting away) or aspiration pneumonia initiated by food going down "the wrong way" into the lungs. TSD is due to deficiency of an enzyme (a protein needed to catalyse a specific chemical reaction within the body). Lack of the enzyme which results in failure to process a lipid (a fat) which accumulates and is deposited in the brain and other tissues, to their detriment. The enzyme is called hexosaminidase-A (hex-A) and the lipid that is deposited is called GM2-ganglioside. TSD is a model of a fatal metabolic disease that occurs primarily within a well- defined subpopulation. It is one of several genetic diseases found more often in persons of Jewish origin. (Other Jewish genetic diseases include Gaucher disease, Niemann-Pick disease, Bloom syndrome, and factor XI deficiency). The frequency of TSD is much higher in Ashkenazi Jews (of European origin) than in other groups of Jews. (In the U.S., 95% of Jews are Ashkenazi and are at risk for TSD). TSD occurs more rarely, in non-Jews. Knowledge of the biochemical basis TSD has permitted screening programmes for carrier detection and prenatal diagnosis of TSD. There are forms of TSD with somewhat more hex-A and hence later onset, termed juvenile TSD and adult TSH. Alternative names for TSD itself are amaurotic familial idiocy

(outdated), B variant GM2-gangliosidosis, hexosaminidase A deficiency, hex-A deficiency. TSD is named for the English physician Waren Tay (1843-1927) and the New York neurologist Bernard (Barney) Sachs (1858-1944). Tay in 1881 studied an infant with progressive neurological impairment and described "symmetrical changes in the yellow spot in each eye", the "cherry-red spots" characteristic of TSD. Sachs saw a child In 1887 and the child's sister in 1898 with the cherry-red spots and "arrested cerebral development" and in 1910 he demonstrated the presence of accumulated lipid in the brain and retina (http://www.medterms.com/script/main/art.asp?articlekey=6654).

APPENDIX



Cell cultures

Method for the establishment of primary fibroblast cell cultures

1. Add 0,5 ml pre-incubated media in a petri dish.
2. Add another 1 ml pre-incubated media in a clearly marked 25 cm^2 flask.
3. Cut the skin biopsy into 1 mm^2 parts and places it onto the bottom of the 25 cm^2 flask with the skin to the top.
4. Incubate the 25 cm^2 flask for 15 min upright so that the biopsies can stick to the surface.
5. Rotate the flask carefully and incubate for 4days.
6. Change medium with 1,5 ml media
7. After two weeks the epithelial cells become visible, were after the fibroblast cells appear.
8. Change media weekly with 4 ml fresh media
9. When the flask is full, cells are split 1:2 and grew for stock freeze

Method for the defrosting of archive stored fibroblast cells

1. Take the sample from the liquid nitrogen
2. Place sample in a water bath at 37 ° C
3. As soon as the sample is fully defrosted, take the sample from the water bath, spray the outside of tube with 70 % ethanol and work in a flow cabinet to ensure sterility.
4. Remove the contents of the tube and place it together with 10 ml warm medium in a bigger tube. Mix the sample with the medium by inverting the tube a few times.
5. Centrifuge the cells at 25 ° C for 2 minutes at 280 X g.
6. The cells are then sedimented at the bottom of the tube. Discard the medium and add fresh media.
7. Mix gently and pipette the mixture in a cell culture flask. Incubate for 2 days or till confluent at 37 ° C, 5 % CO_2.
8. After 2 days, medium should be change.

Media change and trypsinazation

It is important to change the medium at least twice per week depending on the growth tempo of the specific cell culture. If this is not done it can lead to the lowering of the pH, which can result in a negative change of the cell growth.

Media change

1. Discard the old media, wipe the flask opening with 70 % ethanol wetted paper
2. Add 4 ml fresh media
3. Replace the lid, not to tight.
4. Incubate at 37 ° C, 5 % CO_2 till cell harvesting

Trypsinazation

1. Spray the outside of the cell cultured flask with 70 % ethanol and place it in the flow cabinet
2. Discard the old medium, wipe the opening of the flask with 70 % ethanol wetted paper.
3. Add 5 ml PBS to the flask and wash the cells
4. Decant the PBS from the flask and add 1 ml trypsin to the cells. Place the lid back onto the flask and tighten it
5. Incubate for 5 minutes. Shake vigorously to loosen the last clinging cells.
6. Add 7 ml medium to stop the effect of trypsin.
7. Mix the added medium and the cells well. Transport 4 ml cell – medium mix to a new flask.
8. Place the lid onto the flask but look out for not closing it to tight
9. Incubate at 37 ° C, 5 % CO_2 till confluency is reached and it can be stored for later use.

Harvesting of cultured fibroblast cells

1. Spray the outside of the cell cultured flask with 70 % ethanol and place it in the flow cabinet
2. Discard the old medium, wipe the opening of the flask with 70 % ethanol wetted paper.
3. Add 5 ml PBS to the flask and wash the cells
4. Decant the PBS from the flask and add 1 ml trypsin to the cells. Place the lid back onto the flask and tighten it
5. Incubate for 5 minutes. Shake vigorously to loosen the last clinging cells.
6. Add 9 ml medium to stop the effect of trypsin.
7. Mix the contents of the flask well by pipetting
8. Place the contents in a 15 ml centrifuge tube and centrifuge at 280 g for 2 min at 25 °C
9. Decant the medium and add 1,5 ml PBS wash the cells, pepetting it into 1,5 ml centrifuge tubes, centrifuge 280 g for 2 min at 25 °C.
10. Decant the PBS and make sure the pellet remains in the tube

11. Store the dry pellet at –70 °C

Archive storage of cultured fibroblast cells

1. Spray the outside of the cell cultured flask with 70 % ethanol and place it in the flow cabinet.
2. Discard the old medium, wipe the opening of the flask with 70 % ethanol wetted paper.
3. Add 5 ml PBS to the flask and wash the cells
4. Decant the PBS from the flask and add 1 ml trypsin to the cells. Place the lid back onto the flask and tighten it
5. Incubate for 5 minutes. Shake vigorously to loosen the last clinging cells.
6. Add 9 ml medium to stop the effect of trypsin.
7. Mix the contents of the flask well by pipetting
8. Place the contents in a 15 ml centrifuge tube and centrifuge at 280 g for 2 min at 25 °C
9. Decant the medium and add 1,5 ml freeze media and resuspend the cells by pipetting the mixture
10. Pipette the cell freeze medium into a 1,5 ml cryotube.
11. Place the lid on the tube and tight it properly
12. Store the dry pellet at –70 °C for 24 hours
13. After 24 hours place the tube in the nitrogen can for archive storage

APPENDIX

Culturing medium Formulas

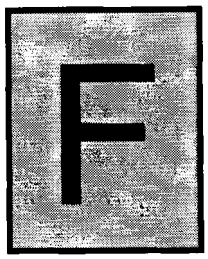


TABLE F1: Chang D Medium® Formula

Components	
	T105
Salts	8515
Glucose	880
Amino Acid	802
L-Glutamine	259
Polypeptides	189
Vitamins	61
Deoxyribonucleosides	35
Ribonucleosides	35
Sodium Pyrovate	97
Other Components	9
Phenol Red	8.8
Hormones and Trace Elements	-
Steroid Hormones	0.0009
Newborn Calf Serum	-
Fetal Bovine Serum	-
Bovine Serum Proteins	12%v/v

TABLE F2: Dulbecco's modified Eagles medium (DMEM)

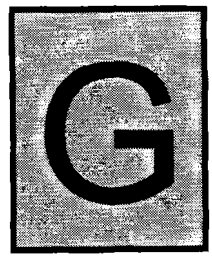
Component	mg/L
$CaCl_2$	200
$Fe(NO_3)_3.9H_2O$	0.1
KCL	400
$MgSO_4$	-
$MgSO_4.7H_2O$	200
NaCl	6400
$NaHCO_3$	3700
NaH_2PO_4	-
$NaH_2PO_4.H_2O$	125
Glucose	4500
HEPES	
Phenol Red	15
Sodium Pyruvate	110
L-Arginine.HCl	84
L-Cystine	48
L-Cystine.2HCl	-
L-Glutamine	584
L-Alanyl-L-Glutamine (UltraGlutamine1)	-
Glycine	30
L-Histidine.HCl.H_2O	42
L-Isoleucine	104.80
L-Leucine	104.80
L-Lysine.HCl	146.20
L-Methionine	30
L-Phenylalanine	66
L-Serine	42
L-Threonine	95.20
L-Tryptophan	16
L-Tyrosine	72
L-Tyrosine.2Na	-
L-Valine	93.60
D-Ca Pantothenate	4
Choline Chloride	4
Folic Acid	4
i-Inositol	7
Nicotinamide	4
Pyridoxine.HCl	4
Riboflavin	0.4
Thiamine.HCL	4

(Dulbecco and Freeman, 1959)

Amniochrome System II

Data sheet not available

APPENDIX



Buffer solutions

These buffer solutions were all prepared according to Sambrook and Russell 2001.

AGAROSE GEL ELECTROPHORESIS

50 X TAE buffer (1L)

242 g	Tris base
57,1 ml	Glacial acetic acid
100 ml	0,5 M EDTA (pH 8,0)
To 1 litre	ddH_2O

1 X TAE buffer (1 L)

20 ml	50 X TAE buffer
To 1L	ddH_2O

0,5M EDTA (pH8,0, 1L)

186,12 g	EDTA
To 1L	ddH_2O

pH 8,0 with 5 N NaOH

5 N NaOH (500 ml)

100 g	NaOH
To 500ml	ddH_2O

1 % Agarose gel Gel size 10 x 14 x 0,7 cm

0,4 g	Agarose
40 ml	1 x TAE buffer
4 µl	Ethidium bromide

Running buffer 1 X TAE buffer

Ethidium Bromide (10mg/ml) **Wrap container in aluminum foil**

0,1 g	Ethidium bromide
10 ml	H_2O

6 X gel-loading dye

25 mg	Bromophenol blue (0,25 % (w/v) bromophenol blue)
25 mg	Xylene cyanol FF (0,25 % (w/v) (xylene cyanol FF)
3 ml	100 % Glycerol (30% (v/v) glycerol in H_2O)
To 10 ml	sterile ddH_2O

FORMALDEHYDE AGAROSE GEL ELECTROPHORESIS

1,2 % Formaldehyde denaturing gel (Gel size approximately 10 x 14 x 0,7 cm)

1,2 g	Agarose
10 ml	10 X FA gel buffer
to 100 ml	Rnase-free H_2O

10 X Formaldehyde (FA) buffer (pH 7,0; 1L)

41,86 g	MOPS (3-[N-morpholino] propanesulfonic acid) (200 $mM_{[Final]}$)
3,102 g	Sodium acetate (50 $mM_{[Final]}$)
3,72 g	EDTA (10 $mM_{[Final]}$)
	5 N NaOH to pH 7,0
To 1L	RNase-free ddH_2O

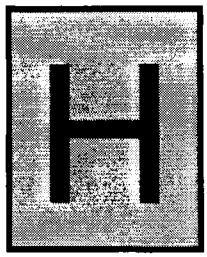
1 X Formaldehyde (FA) running buffer (1L)

100 ml	10 X FA gel buffer
20 ml	37 % (12,3 M) formaldehyde
To 1L	RNase-free ddH_2O

5 X RNA loading buffer (10ml)

16 µl	saturated aquese bromophenol blue solution
80 µl	500 mM EDTA (pH 8,0)
720 µl	37 % (12,3 M) formaldehyde
2 ml	100 % glycerol
3084 µl	formamide
4 ml	10 X FA gel buffer
To 10 ml	RNase-free ddH_2O

APPENDIX



DNA isolation: Flexigene® (Qiagen)

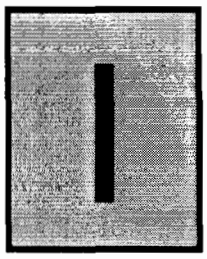
Isolation of genomic DNA from cultured fibroblast cells and with Flexigene®

1. Thaw cells quickly in a 37 °C warm bath with mild agitation and stored on ice before beginning the procedure.
2. Add 300 µl Buffer FG1 (lyses buffer) to the cell pellet and vortex until the cells are resuspending.
3. Mix 300 µl of Buffer FG2 (denaturation buffer) and 3 µl of QIAGEN protease
4. Add the FG2 and protease mixture to the FG1 (lyses buffer) cell mixture, vortex lightly and incubate at 65 °C for 10 min.
5. Centrifuge for 2 min full speed
6. Carefully pippette the upper DNA containing layer to a clean 1, 5 ml tube. Avoid pipettng the pellet or the liquid at the bottom of the tube. *(This step was inserted to pellet the unlysed cells at the bottom and to prevent protein contamination previously experienced.)*
7. Add 600 µl 100% isopropanol and mix thoroughly by inversion until the DNA precipitate becomes visible as threads of a clump.
8. Centrifuge for 3 min at 13 000 rpm.
9. Discard the supernatant and take care that the pellet remains in the tube (DNA should be visible as a small white pellet).
10. Add 600 µl 70 % ethanol and vortex lightly.
11. Centrifuge for 3 min at 13 000 rpm.
12. Discard the supernatant and take care that the pellet remains in the tube.
13. Air-dry the DNA pellet until all the liquid has evaporated (5 min).
14. Add 200 µl Buffer FG3 (rehydration buffer), vortex briefly at a low speed.
15. Incubate at 65 °C for 40 min. Incubation time may need to be prolonged.
16. Store at 4 °C for 48 hours before used as template in PCR reactions or gels.

Isolation of genomic DNA from buffy coat with Flexigene®

1. Store buffy coat (1,0 ml per 1,5ml tube) at –70 °C. / prepare buffy coat from fresh blood collected in a heparin coated tube.
2. Thaw cells quickly in a 37 °C warm bath with mild agitation and stored on ice before beginning the procedure.
3. Add 500 µl ice cold PBS solution. Centrifuge 10 min at full speed.
4. Discard supernatant, take care that the pellet remains in the tube.
5. Add 1250 µl Buffer FG1 (lyses buffer) to the cell pellet and vortex until the cells are resuspend.
6. Mix 500 µl of Buffer FG2 (denaturation buffer) and 5 µl of QIAGEN protease
7. Add the FG2 and protease mixture to the pelleted cells, vortex until the pellet is completely homogenized and incubate at 65 °C for 5 min.
8. Add 500 µl 100% isopropanol and mix thoroughly by inversion until the DNA precipitate becomes visible as threads of a clump.
9. Centrifuge for 3 min at 10 000 rpm.
10. Discard the supernatant and take care that the pellet remains in the tube (DNA should be visible as a small white pellet).
11. Add 500 µl 70 % ethanol and vortex lightly.
12. Centrifuge for 3 min at 10 000 rpm.
13. Discard the supernatant and take care that the pellet remains in the tube.
14. Air-dry the DNA pellet until all the liquid has evaporated (5 min).
15. Add 200 µl Buffer FG3 (rehydration buffer), vortex briefly at a low speed.
16. Incubate at 65 °C for 40 min. Incubation time may need to be prolonged.
17. Store at 4 °C for 48 hours before used as template in PCR reactions or gels.

APPENDIX



RNA isolation: RNeasy®and Qiazol® (Qiagen)

Isolation of total RNA from cultured fibroblast cells with RNeasy®

1. Harvest cells
2. One 25 cm^2 flask of cells (<5 X 10^6) is used in one reaction.
3. Frozen cells must be thaw slightly.
3. Disrupt cells by addition of 350 µl Buffer RLT.
4. Homogenize cells for 30 s using a rotor-stator homogenizer (vortex).
5. Add 350 µl of 70 % ethanol to the homogenized lysate and mix well by pipetting.
6. Do not centrifuge.
7. Apply up to 700 µl of the sample, including any precipitate that may have formed to an RNeasy® mini column placed in a 2 ml collection tube (supplied), close the tube gently.
8. Centrifuge for 15 s at 10 000 rpm.
9. Discard the flow through.
10. Add 700 ul Buffer RW1 to the RNeasy® column. Close the tube gently.
11. Centrifuge for 15 s at 10 000 rpm.
12. Discard the flow through.
13. Transfer the RNeasy® column into a new collection tube.
14. Pitet 500 µl Buffer RPE onto the RNeasy® column, close the tube gently.
15. Centrifuge for 15 s at 10 000 rpm.
16. Discard the flow through.
17. Add another 500 µl Buffer RPE to the RNeasy® column, close the tube gently.
18. Centrifuge for 15 s at 10 000 rpm.
19. Transfer RNeasy column in a new 1,5 ml collection tube (supplied).
20. Pipet 40 µl RNase free water directly onto the RNeasy silica-gel membrane, close the tube gently.
21. Centrifuge for 1 minute at 10 000 rpm.

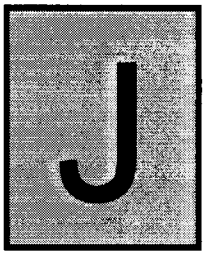
Total RNA Cleanup using RNeasy®

1. Adjust sample to a volume of 100 µl with RNase-free water.
2. Add 350 µl Buffer RLT and mix thoroughly
3. Add 250 µl ethanol (96 -100 %) to the diluted RNA, and mix thoroughly by pipetting *Do Not centrifuge*
4. Apply 700 µl of the sample to an Rneasy mini column placed in a 2 ml collection tube. Close the tube gently and centrifuge for 15 s at 10 000 rpm
5. Transfer RNeasy column in a new 1,5 ml collection tube (supplied).
6. Pipet 500 µl Buffer RPE onto the RNeasy® column, close the tube gently and centrifuge for 15 s at 10 000 rpm to wash the column and Discard flow-through
7. Add another 500 µl Buffer RPE onto the RNeasy® column, close the tube gently and centrifuge for 2min at 10 000 rpm
8. Place column in a new 2 ml collection tube and centrifuge full speed for 1 min
9. Transfer the Rneasy column to a new 1,5 ml collection tube. Pipet 40 µl RNase free water directly onto the RNeasy silica-gel membrane. Close the tube gently, and centrifuge for 1 min at 10 000 rpm

Total RNA isolation with Qiazol®

1. Harvest cells
2. One 25 cm^2 flask of cells (<5 X 10^6) is used for every 2 ml of Qiazol® (1 Volume).
3. Frozen cells must be thaw slightly.
4. Homogenize cells for 30 s using a rotor-stator homogenizer (vortex) and a 20 gage needle and syringe.
5. Add a 1/3 volume of chloroform to the homogenised cells
6. Vortex and shake vigirously
7. Leaf at room temperature for 10 min. A water layer form at the top of the mixture
8. Centrifuge for 30 min at maximum speed
9. Phase separation took place. Transfer the upper aquase phase to a new tube
10. Add ½ volume of isopropanol to the aquaes phase.
11. Vortex and put on ice for 10 min
12. Centrifuges for 30 min at maximum speed
13. Discard the isopropanol but take care that the pellet remain in the tube
14. Add 500 µl of ice cold 70 % ethanol to the pellet and vortex
15. Centrifuge for 2 min at maximum speed
16. Discard the supernatant and left the pellet to dry
17. Add 30 -50 µl RNase free water to the pellet

APPENDIX



Materials and reagents

TABLE J1: CONSUMABLES AND OTHER MATERIALS

Item	Catalog number	Manufacturer
10 ml Pipette	PN10E1: M14M0943001	Whitehead Sci
10µl Filter tips	1051965038	Labcon
100 -1000µl Filter tips	1057965108	Labcon
100 -1000µl Tips	1041960008	Labcon
200µl Filter tips	1059965008	Labcon
200µl Tips	1030965008	Labcon
22G X 11/4' Needles	0123	-
40µl Filter tips	1055965018	Labcon
Carbon blades	0434	Paramount
Centrifuge tubes (1,5 ml)	3016-087-000	Labcon
Centrifuge Tubes (15 ml)	3131-345-008	Labcon
Cryogenic vials 1,2 ml	430659	Corning
Cryogenic vials 2ml	430658	Corning
Flasks (25 cm^2)	90025	TPP
Flasks (75 cm^2)	90075	TPP
Guthrie cards	WB100014	Whatman
Heparin coated blood collecting tubes	BD-LH100.0U	Belliver Industrial Setate
Latex gloves	Happy skin	Umar
PCR tubes (0,2 ml)	3936500000	Labcon
Plastic Pasteur pipettes	Liquipette	Elkay
Syringe 1 ml	0197`	Terumo

TABLE J2: CHEMICALS, REAGENTS AND KITS

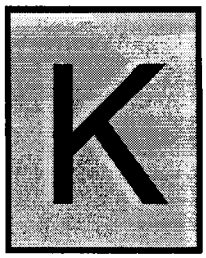
Item	Catalog number	Manufacturer
50 bp Ladder	SM0371	Fermentas
Absolute ethanol	1009832500	Merck
Agarose	D1LE	Hispanger
Amniochrome system II	BE12-756F	BioWhittaker
Bromophenol blue	064/0811B	BDH
Chloroform	C2432-500	Sigma-Aldrigh
Cloned AMV	12328-019	Invitrogen
Dimethylformamide	BB103226L	BDH
DMEM	41966-029	Gibco
DMSO	D2650-100ml Hydrimax	Sigma-Aldrigh
dNTPs (25mM each)	DM105B	Bioline
EDTA	03685	Fluka
Ethidium bromide	200-271	Roche
Fetal bovine serum	10108165	Gibco
Fetal calf serum		
Flexigene® Genomic DNA isolation kit	51206	Qiagen
Formaldehyde (37 %, 12,3M)	47629	Fluka
Gel extraction Kit	28706	Qiagen
Gentamycin	17-5182	BioWhittaker
Glacial acetic acid	BB100017P	BDH
Glycerol	1040921000	Merck
Histopaque®	1071	Sigma-Aldrich
HPLC purified water	152736D	BDH
Isopropanol	BB1022246L	BDH
MOPS	11124684001	Roche
NaOH pellets	1064980500	Merck
O'gene ruler	SM1173	Fermentas
Parafilm	PM996	Parafilm
PBS	BR0014G	Oxoid
Pen/strep	17-602E	BioWhittaker

Phusion DNA polymerase	F530L	Finnzymes
Primers		www.metabion.com www.tibmolbiol.com ROCHE
Qiazol®	79306	Qiagen
RNA*later*	7020, 7021	Ambion
RNasin	N251A	Promega
RNeasy® RNA isolation kit	74104	Qiagen
Sodium acetate	71183	Fluka
TaKaRa Ex Taq DNA polymerase	RR001	TaKaRa
TaKaRa Taq DNA polymerase	R001A	TaKaRa
TaKaRa Taq DNA polymerase $MgCl_2$ free buffer	R001AM	TaKaRa
Tripsin-EDTA	15400-054	Gibco
Tris-base	11814273001	Roche
Xylene Cyanol FF		
β-Mercaptoehanol	44143	BDH

TABLE J3: APPLIANCES

Item	Catalog number	Manufacturer
- 20 °C Freezer	-	Kalvinatior
- 80 °C Freezer	-	Vacuted
15 ml Tube centrifuge	Rotofix 32	Hettich
4 °C Refrigerator	-	Kalvinatior Classic
Chromas	Free software	Free software
Dark reader	DR-088M transilluminator	Clare Chemical research
Flow cabinet	Bio Flow	Labotec
Geldoc	SYNGENE	Vacutec
Genesnap	SYNGENE	Vacutec
Heating block	QBD2	Grant
CO_2 Incubator	Hera Cell	Hera Cell
Light microscope	DM/L 090135002	Leica
Mastercycler	534T 007499	Eppendorf
Mega	Free software	Free software
Nanodrop	ND1000	Fermentas
Pipette 10µl	Termo	Finnpipette
Pipette 1000 µl	Termo	Finnpipette
Pipette 200 µl	Termo	Finnpipette
Pump	-	Flow laboratories
Scale	BRB54	Boeco
Table centrifuge	5415D	Eppendorf
Vectro NTI	Free software	Invitrogen
Vortex	REAX1 5411	Heindolph

APPENDIX



Sequencing Requirements

Bad quality DNA results in bad sequences gained during sequence detection. Therefore was a summary of the requirements made to ensure good quality sequencing. The sequencing laboratory of the Onderstepoort Vetenering Institute used the ABI3100 Genetic Analyser to determine the sequences of DNA fragments.

OVI Guidelines for sample preparation

X ul DNA

1 ul of a 3,2 pmol primer

RNase and DNase free water to 12 ul

X is determined by the amount of PCR Product obtained during PCR. The final quantity DNA necessary to determine the sequence of a specific length amplicons is tabled in table K1.

TABLE K1: RECOMMENDED DNA QUANTITIES

Single stranded DNA	50 – 100 ng
Double stranded DNA	200 – 500 ng
PCR Product	
<500 bp	3 -10 ng
500 -1000 bp	5 - 20 ng
1000 – 2000 bp	10 -40 ng
> 2000 bp	40 – 100 ng

Inqaba biotech guidelines for sample preparation

Recommendation for sequencing at inqaba is tabled in table K2. Inqaba determine the concentration of each product and the reactions was done as according the quality/quantity of the DNA product.

TABLE K2: RECOMMENDATIONS OF INQABA

ECO-BASIC	25 ul Gel extracted PCR product
	10 ul 10 uM Primer
FULL SERVICE	25 ul Raw PCR product
	10 ul 10 uM Primer

APPENDIX

Biochemical profiles

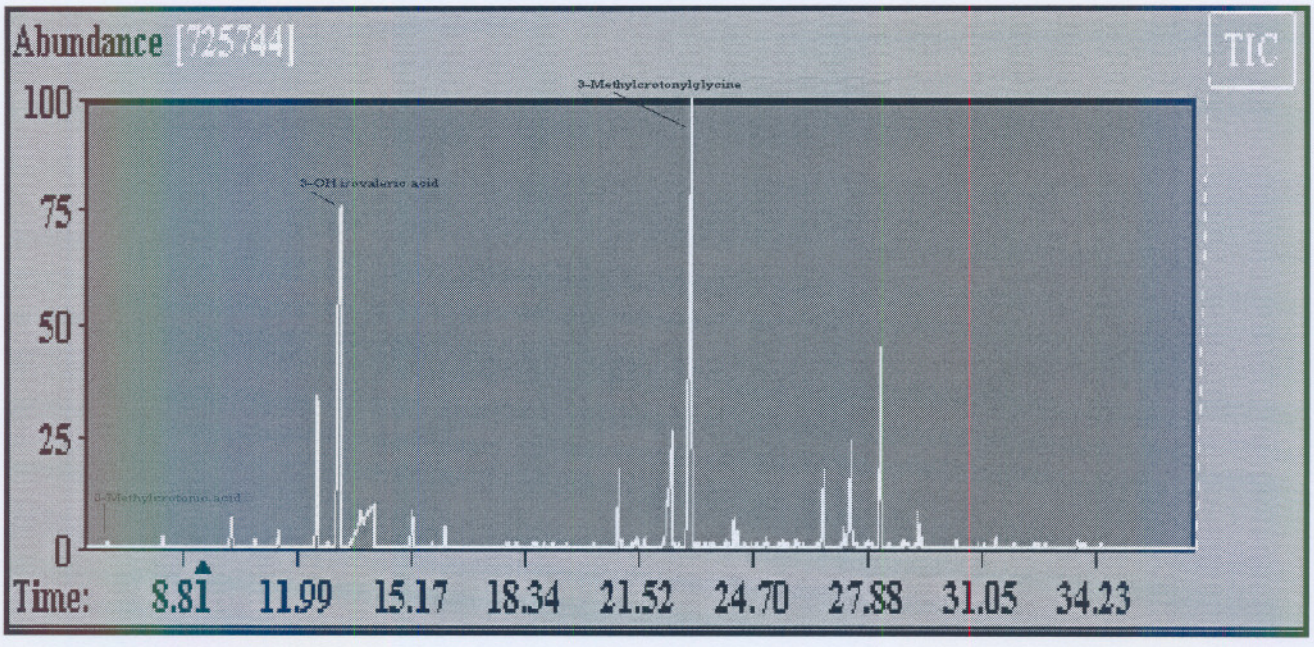


FIGURE L1: GC/MS ANALYSES OF A MCC DEFICIENT PATIENT PROFILE

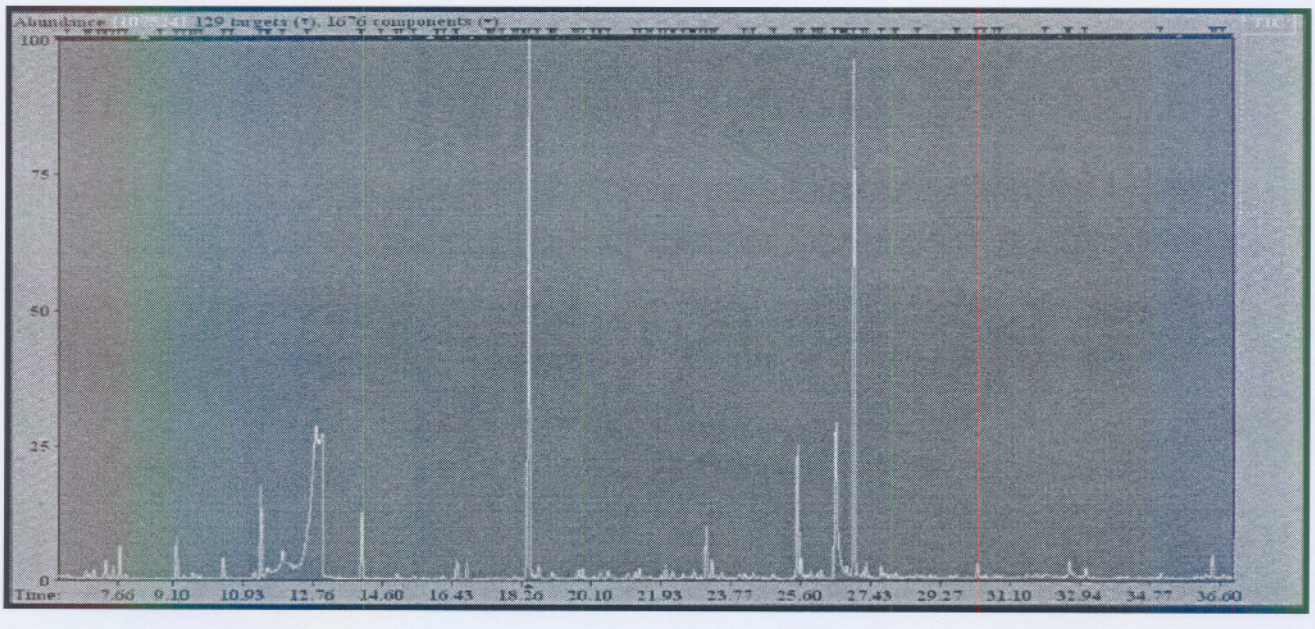
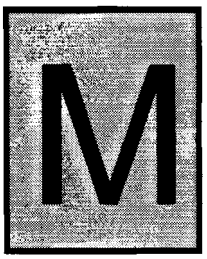


FIGURE L3: NORMAL GC/MS PROFILE

APPENDIX



Qiaquick® Gel extraction (Qiagen)

1. Prepare a preparative 1 % agarose gel.
2. Load 45 µl sample with 10 µl loading dye and electrophoresed for 60 min at a constant voltage of 70 V.
3. Carefully cut the correct band from the gel using a scalpel
4. Weigh the gel slice.
5. Add 3 volumes of Buffer QC to 1 volume of gel (100 mg – 100 µl)
6. Incubate at 50 °C for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, vortex tube 2-3 times during incubation

If the color of the mixture is orange or violet add 3 M sodium acetate pH 5 and mix. The color of the mixture will return to yellow.

7. Add 1 volume of isopropanol to the sample and mix
8. Place a Qiaquick® spin column in a provided collection tube
9. Apply the sample to the column and centrifuge for 1 min 13 000 rpm
10. Discard flow-through and repeat if necessary.
11. Discard flow-through and add 500 µl Buffer QC to Qiaquick® column and centrifuge for 1 min at 13 000 rpm
12. Discard flow-through and add 750 µl BufferPE to Qiaquick® column and centrifuge for 1 min at 13 000 rpm
13. Discard flow-through and centrifuge for an additional 1 min at 13 000 rpm
14. Place column in a clean 1,5 ml tube add 50 µl Buffer EB to the centre of the membrane and centrifuge for 1 min.