A Novel Mitochondrial DNA (mtDNA) Mutation Significantly Attenuates Transcription Termination In A Patient With A Mitochondrial Myopathy

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A thesis submitted for the degree of Doctor of Philosophy

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Adelaide, South Australia.
The scientist has a lot of experience with ignorance and doubt and uncertainty, and this experience is of very great importance, I think.

It is scientific only to say what is more likely and what less likely, and not to be proving all the time the possible and impossible.

Our imagination is stretched to the utmost, not, as in fiction, to imagine things which are not really there, but just to comprehend those things which are there.

Richard Feynman

What is science in the last analysis but the study and the love of Nature, displayed not in the form of abstract worship but in the practical form of seeking to understand Nature?

... the principal requisite for success in scientific research is not the maturity of knowledge associated with age and experience, but the freshness of outlook which is the natural attribute of youth.

Sir C.V.Raman

“...what a long strange trip it’s been”

The Grateful Dead
Dedication

I dedicate this thesis to the memory of my mother, Lalitha Raghupathi (1941-2001) and my maternal grandmother, G. Ambujammal (1918-2010).
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Declaration

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge or belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Ravinarayan Raghupathi
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I wish to place on record my gratitude to the Government of Australia and Flinders University for the Endeavour International Postgraduate Research Scholarship, without which this study would have been impossible. I thank the Departments of Human Physiology and Neurology for giving me the opportunity to undertake my PhD study.

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I would be remiss if I did not acknowledge the proband, whose cells and tissues formed the basis of this study. I had the pleasure of interacting with him on several occasions and I wish him well.

I would never have come this far were it nor for the support of my parents, (Varadachar and Lalitha Raghupathi), my maternal grandmother and my family and friends. I am deeply grateful to all of them for their love and support, especially when the going got tough.

There are no words to really describe my gratitude to my wonderful wife, Dr. Anuradha Mundkur, without whom my life would not have been the same. Her
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<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Description</th>
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<tr>
<td>8-OHdG</td>
<td>8-hydroxy-2-deoxyguanosine</td>
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<td>AD</td>
<td>Alzheimer’s Disease</td>
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<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ADPD</td>
<td>Alzheimer’s Disease and Parkinson’s Disease</td>
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<tr>
<td>adPEO</td>
<td>Autosomal Dominant Progressive External Ophthalmoplegia</td>
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<tr>
<td>ALS</td>
<td>Amyotrophic Lateral Sclerosis</td>
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<tr>
<td>ALT</td>
<td>Alanine transaminase</td>
</tr>
<tr>
<td>AP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate aminotransferase</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BN-PAGE</td>
<td>Blue-Native Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>bp</td>
<td>Base-pair</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>Calcium chloride</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
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<tr>
<td>CK</td>
<td>Creatine kinase</td>
</tr>
<tr>
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<td>Central Nervous System</td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme A</td>
</tr>
<tr>
<td>CoQ</td>
<td>Coenzyme Q</td>
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<tr>
<td>COX</td>
<td>Cytochrome c oxidase</td>
</tr>
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<td>CPEO</td>
<td>Chronic progressive external ophthalmoplegia</td>
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<tr>
<td>CRS</td>
<td>Cambridge Reference Sequence</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>CS</td>
<td>Citrate synthase</td>
</tr>
<tr>
<td>CSB</td>
<td>Conserved Sequence Blocks</td>
</tr>
<tr>
<td>DIG</td>
<td>Digoxigenin</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5’-dithiobis(2-nitrobenzoic acid)</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra-acetic acid</td>
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<tr>
<td>EEG</td>
<td>Electroencephalography</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>EtBr</td>
<td>Ethidium bromide</td>
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<tr>
<td>ETC</td>
<td>Electron Transport Chain</td>
</tr>
<tr>
<td>FMN</td>
<td>Flavin mononucleotide</td>
</tr>
<tr>
<td>GGT</td>
<td>gamma-glutamyl transferase</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HD</td>
<td>Huntington’s Disease</td>
</tr>
<tr>
<td>HMG</td>
<td>High mobility group</td>
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<td>Hsp</td>
<td>Heat shock protein</td>
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<td>HSP</td>
<td>Heavy strand promoter</td>
</tr>
<tr>
<td>IMS</td>
<td>Inter-membrane space</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium chloride</td>
</tr>
<tr>
<td>KCN</td>
<td>Potassium cyanide</td>
</tr>
<tr>
<td>KSS</td>
<td>Kearns-Sayre syndrome</td>
</tr>
<tr>
<td>LHON</td>
<td>Leber’s Hereditary Optic Neuropathy</td>
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<tr>
<td>LIMM</td>
<td>Lethal Infantile Mitochondrial Myopathy</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
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<tr>
<td>LSP</td>
<td>Light strand promoter</td>
</tr>
<tr>
<td>MELAS</td>
<td>Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like symptoms</td>
</tr>
<tr>
<td>MERRF</td>
<td>Myoclonic Epilepsy and Ragged-Red Fibre Disease</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>MMC</td>
<td>Maternally Inherited Myopathy and Cardiomyopathy</td>
</tr>
<tr>
<td>MND</td>
<td>Motor Neuron Disease</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>mtDBP</td>
<td>Mitochondrial displacement (D)-loop binding protein</td>
</tr>
<tr>
<td>mtDNA</td>
<td>Mitochondrial DNA</td>
</tr>
<tr>
<td>mTERF</td>
<td>Mitochondrial transcription termination factor</td>
</tr>
<tr>
<td>MTG</td>
<td>MitoTracker Green</td>
</tr>
<tr>
<td>mt-Hsp</td>
<td>matrix heat shock protein</td>
</tr>
<tr>
<td>mtRNA</td>
<td>Mitochondrial RNA</td>
</tr>
<tr>
<td>MTS</td>
<td>Matrix targeting sequences</td>
</tr>
<tr>
<td>mtSSB</td>
<td>mitochondrial single-stranded binding protein</td>
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<tr>
<td>mtTFA/TFAM</td>
<td>Mitochondrial transcription factor A</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>reduced Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADH-TR</td>
<td>NADH-tetrazolium reductase</td>
</tr>
<tr>
<td>NARP</td>
<td>Neurogenic ataxia retinitis pigmentosa</td>
</tr>
<tr>
<td>NCR</td>
<td>Non-coding regions</td>
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<tr>
<td>nt</td>
<td>Nucleotide</td>
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<td>OXPHOS</td>
<td>Oxidative phosphorylation</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
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<tr>
<td>PAM</td>
<td>Presequence Translocase Associated Motor</td>
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<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<td>Polymerase chain reaction-restriction fragment length polymorphism</td>
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<td>PCR-RSM</td>
<td>PCR-mediated restriction site modification</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s Disease</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly-ethylene glycol</td>
</tr>
<tr>
<td>PEM</td>
<td>Progressive encephalomyopathy</td>
</tr>
<tr>
<td>POLRMT/mtRPOL</td>
<td>Mitochondrial RNA Polymerase</td>
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<tr>
<td>PPR</td>
<td>Pentacotripeptide Repeat</td>
</tr>
<tr>
<td>PTP</td>
<td>Permeability Transition Pore</td>
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<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
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<tr>
<td>qPCR</td>
<td>Quantitative PCR</td>
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<tr>
<td>REST</td>
<td>Relative Expression Software Tool</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>RRF</td>
<td>Ragged-Red Fibres</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal RNAs</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse Transcriptase-Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SAM</td>
<td>Sorting and assembly machinery</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDH</td>
<td>Succinate dehydrogenase</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>SSC</td>
<td>Saline sodium citrate</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris acetate EDTA</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris borate EDTA</td>
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<tr>
<td>TIM</td>
<td>Translocase of the inner membrane</td>
</tr>
<tr>
<td>TOM</td>
<td>Translocase of the outer membrane</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer RNA</td>
</tr>
<tr>
<td>Wt</td>
<td>Wild type</td>
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Abstract

This PhD study aimed to characterise the pathophysiology of a novel mitochondrial mutation in a patient with a mitochondrial myopathy. This mutation, an adenine insertion at nucleotide (nt) position 3230 of the human mitochondrial genome (RefSeq NC_012920), was shown to significantly disrupt transcription termination, leading to increases in the levels of both the genome-length mitochondrial DNA (mtDNA) polycistronic transcript and selected mRNAs encoding subunits of the respiratory chain complexes. A corresponding increase in the levels of two subunits of Cytochrome c oxidase (COX, Complex IV) was observed; however, there was no increase in the levels of any of the respiratory chain holocomplexes. Complex I and Complex IV activities were elevated in the proband’s tissues. No evidence of DNA damage through apoptosis or necrosis was found and the proband’s cells did not show elevated levels of 8-hydroxy-2-deoxyguanosine, a biomarker of oxidative stress. Attenuation of transcription termination in human mitochondria appears to be a novel mechanism of mitochondrial disease.
This thesis is divided into six chapters. Chapter I provides a comprehensive review of pertinent literature in the field of human mitochondrial disease. It covers the biogenesis of mitochondria, mitochondrial function and dysfunction and explores the approaches commonly used in the study of mitochondrial disease. It ends with the background to this project, including the clinical case study, and outlines the aims of the project.

Chapter II details the biological samples used in this study, which included lymphoblasts, cytoplasmic hybrids (cybrids) and skeletal muscle biopsy samples obtained from the proband and six controls and the experimental procedures used to obtain the results described.

In Chapter III, the initial molecular genetic analysis of the mutation is described. Using a PCR-RFLP assay, the pedigree and tissue distribution of the mutation and its load in different samples was studied. Mitochondrial mass and number were assayed using a mitochondrion-selective fluorophore, MitoTracker Green. The effect of this mutation on tRNA^{Leu(UUR)} folding was analysed using mFOLD.

Chapter IV describes the study of the effects of the mutation on mtDNA transcription and translation. The levels of the polycistronic transcript and three mtDNA mature transcripts (ND1, ND2 and COX 1) were measured by Real-time RT-PCR and Northern blotting. Respiratory chain holocomplex levels were analysed by Blue-Native PAGE and mass spectrometry. The levels of COX sub-
units I, II and IV were measured by immunoblotting with monoclonal antibodies against each subunit.

The penultimate Chapter (V) details the study of the effects of this mutation on mitochondrial function. Standardised spectrophotometric assays were used to measure respiratory chain activity. DNA damage was analysed by gel electrophoresis to look for the typical DNA laddering associated with apoptosis. A commercial ELISA kit was used to measure 8-OHdG levels.

Chapter VI presents an in-depth discussion of the overall findings and looks at their implication in a novel mechanism of mitochondrial disease in humans. This project has paved the way for interesting future projects, some of which are introduced in this chapter.

Note: unless otherwise mentioned, all artwork presented in this thesis is original.
Chapter I

Introduction and Literature Review
1.1 Introduction

1.1.1 General Introduction

The current trend in scientific research is tending towards an integrative approach to the study of biological systems in health and disease (Beard and Vendelin 2006). Such an approach attempts to unite data sets from genomics, proteomics and metabolomics, as well as mathematical and computational predictions, to diagnose and characterise complex diseases (Vo and Palsson 2007). In the last forty years, there has been increasing evidence for the involvement of defects in the cellular bioenergetics system and mitochondrial function in the etiology of several diseases and disorders (Schapira 1998; Wallace 1999; Scarpulla 2008). Interestingly, the unraveling of the mechanisms of cellular respiration and energy production represents one of the earliest integrated biology approaches, and came about by collaborative “interfield excursions” by cytologists and biochemists (Bechtel and Abrahamsen 2007). These were based on the independent observations by Richard Altmann and Carl Benda in the late 19th century, of a cellular organelle that appeared threadlike at times and granular at others. Benda named them “mitochondria” from the Greek terms for thread (mitos) and granule (chondros) (Ernster and Schatz 1981; Bechtel and Abrahamsen 2007). In 1899, Leonor Michaelis showed that mitochondria were involved in cellular respiration because they selectively bound Janus Green, a dye used to identify oxidation-reduction reactions (Bechtel and Abrahamsen 2007). Over the next seven decades, cytologists and biochemists pieced together the structure and function of mitochondria.
Mitochondria are responsible for energy generation and contain the enzymes of the citric acid cycle, fatty acid oxidation and oxidative phosphorylation (Kennedy and Lehninger 1949). During aerobic respiration, they convert oxygen and other nutrients into the universal energy “currency” ATP (adenosine triphosphate) that powers a cell’s metabolic activities. This process, called chemiosmotic coupling (Mitchell 1961) and reviewed in detail below, involves the transfer of energy-rich electrons along a chain of carriers embedded in an ion-impermeable membrane (Hatefi 1985). Whilst travelling along this “electron-transport chain”, the electrons reach successively lower energy levels; part of the released energy is used to pump protons from one side of the membrane to the other, generating what is referred to as the proton gradient or proton motor force across the membrane (Mitchell 1961).

The number of mitochondria in a cell varies from cell to cell and can change depending on the metabolic requirements of the cell or tissue. For example, mammalian liver cells each contain 1000-2000 mitochondria, making them ideal candidates for biochemical and molecular studies (Ernster and Schatz 1981).

Apart from ATP synthesis, mitochondria contribute to the biosynthesis of pyrimidines, amino acids, nucleotides and many other metabolites (Attardi and Schatz 1988). Among the important intracellular processes they are involved in is the production of reactive oxygen species (ROS), calcium ion regulation and apoptosis. ROS such as the superoxide and hydroxyl radicals, hydrogen peroxide and singlet oxygen are the products of normal metabolism (Chance et al. 1979; Ambrosio et al. 1993) but can be potentially toxic when produced in large
quantities, leading to tissue injury (Ambrosio et al. 1993). Mitochondria use about 98% of cellular oxygen to produce ATP and about 1-2% of it is converted by them into superoxide (Chance et al. 1979). They play a major role in lipid peroxidation and the production of semiquinone radicals (Chance et al. 1979; Richter 1992) and their respiratory function declines with age due to long-term exposure to these free radicals (Richter 1992; Salganik et al. 1994).

Mitochondria also act as high-capacity Ca\(^{2+}\) sinks by transporting the ion from the cytosol for the regulation of dehydrogenases involved in the citric acid cycle, and accumulating it in high concentrations (Nicholls and Budd 2000; Nicholls 2005). The transport of calcium into and out of the mitochondrion is effected by specific proteins and increased Ca\(^{2+}\) loading causes the formation of a mitochondrial permeability transition pore (PTP) in the inner membrane (Norenberg and Rao 2007). This pore allows large solutes to enter the mitochondrial matrix, causing osmotic swelling of the mitochondrion. This in turn dissipates the proton gradient, stops ATP synthesis and initiates the release of apoptogenic factors (Nicholls and Budd 2000; Norenberg and Rao 2007). In fact, recent studies have implicated mitochondria in both apoptosis and necrosis (Kroemer and Reed 2000). Apoptotic outer membrane permeabilisation involves the release of proteins which are normally confined to the intermembrane space of mitochondria, including cytochrome c, certain pro-caspases, adenylate kinase 2 and apoptosis-inducing factor (Kroemer and Reed 2000). Studies with recombinant pro-apoptotic members of the Bcl-2 family of protein have shown that they most likely cause apoptosis by causing the formation of a PTP (Kroemer and Reed 2000; Shults 2004) and it appears that this event most likely marks a point of no return in
apoptosis (Petit et al. 1997). Mitochondrial dysfunction and associated bioenergetic failure can lead to abnormal cellular ion homeostasis, as a result of which cells undergo swelling and cellular disruption, eventually leading to necrotic death (Norenberg and Rao 2007).

1.1.2 Structure of Mitochondria

Mitochondria are usually depicted as stiff, elongated, bacteria-like cylinders (Fig. 1.1) with a diameter between 0.5 and 1µm. However, they are extremely mobile organelles and constantly change their shape, divide and may even fuse with each other (Pollak and Sutton 1980). Each mitochondrion is defined by two highly specialised lipid bilayers – the outer and inner mitochondrial membranes – that play crucial roles in its activities. The compartments formed by these membranes are the narrow intermembrane space and the internal matrix. Each well-defined area of a mitochondrion contains several highly specialised proteins that define its functionality (Darnell et al. 1986; Alberts et al. 1989).

The outer mitochondrial membrane has numerous channel-forming transport proteins called porins – these aqueous channels are permeable to molecules 10 kDa or less in size and especially to protons (DePierre and Ernster 1977; Darnell et al. 1986). Other proteins embedded in this membrane include enzymes that catalyse mitochondrial lipid synthesis and the conversion of lipid substrates into molecules subsequently metabolised in the matrix.
The intermembrane space is used by several enzymes that use the ATP passing out of the matrix to phosphorylate other nucleotides.

The matrix contains a mixture of hundreds of enzymes, many of which play a crucial role in the oxidation of pyruvate and fatty acids in the citric acid (Krebs) cycle, the initial steps in the production of ATP through aerobic respiration. It also contains several identical copies of the mitochondrial genome as well as its products such as ribosomes and tRNAs, in addition to nuclear-encoded transcription factors and enzymes required for the expression of the mitochondrial genome that are imported into the mitochondrial matrix (Srere 1982; Falkenberg et al. 2007).
The inner mitochondrial membrane is highly convoluted and invaginated into “cristae” which greatly increase its surface area (Pollak and Sutton 1980). About 20% of the lipid content of this membrane is composed of the “double” phospholipid cardiolipin (diphosphatidylglycerol), whose chemical nature is thought to make the membrane impermeable to ions (Bishop and Bell 1988; Chicco and Sparagna 2007). This impermeability is important to maintain the electrochemical gradient that drives ATP synthesis in the mitochondrion (Hackenbrock 1981). The inner membrane is the site of the electron transport chain (ETC) and contains proteins with three types of functions (Srere 1982):

(i) respiratory chain oxidative reactions
(ii) ATP synthesis, and,
(iii) metabolite transport into and out of the matrix.

As mentioned earlier, mitochondria are responsible for the culminating stages of aerobic respiration leading to the production of ATP by oxidative phosphorylation. The flow of electrons from various substrates to oxygen occurs through the ETC, which consists of a series of proteins with tightly-bound prosthetic groups capable of accepting and donating electrons in a specific sequence (Lehninger 1982; Hatefi 1985). This link between chemical processes and transport processes gives rise to the concept of “chemiosmotic coupling” (Alberts et al. 1989).
1.1.3 The Electron Transport Chain

Each turn of the citric acid cycle (Krebs cycle) produces four pairs of hydrogen atoms by the action of specific dehydrogenases on isocitrate, α-ketoglutarate, succinate and malate. In addition, a pair each of hydrogen atoms comes from the degradation of pyruvate, fatty acids and amino acids (Lehninger 1982). These hydrogen atoms donate their electrons to the ETC and become H⁺ ions, which escape into the aqueous medium.

The ETC consists of four enzymatic complexes of proteins (Complexes I – IV) that pass along the electrons in a defined sequence (Hatefi 1985; Heales et al.)
Fig. 1.2 shows a schematic of these four complexes while Fig. 1.3 illustrates the hypothesised number of electron carrying groups in each complex. The entry of most of the electron pairs into the ETC comes about by the action of dehydrogenases that use the coenzyme NAD (nicotinamide adenine dinucleotide) (Lehninger 1982; Alberts et al. 1989).

1.1.3.1 Complex I

The first and largest enzyme complex in the ETC is Complex I, NADH-ubiquinone reductase (EC 1.6.5.3) which catalyses the transfer of 2 electrons from NADH to ubiquinone (Hatefi 1985). The first step in this process involves the reduction of FMN (flavin mononucleotide), the tightly bound prosthetic group of
NADH dehydrogenase, which also contains several non-heme iron atoms. These iron-sulphur centres transfer the electron pair to ubiquinone (Coenzyme Q), a fat-soluble quinone found ubiquitously in almost all cells. Mammalian complex I has around 43 sub-units and a molecular mass of approximately 900 kDa (Weiss et al. 1987).

The cytochromes are electron-carrying heme proteins in which the iron is present as a iron-porphyrin prosthetic group similar to hemoglobin. There are three classes of cytochromes – \( a \), \( b \) and \( c \) – which, in the ETC, occur in the sequence \( b-c_1-c-aa_3 \) (Chance and Williams 1955). Cytochrome \( c \) is a small protein of MW 12.5 kDa and is highly conserved amongst most species (Capaldi et al. 1982).

1.1.3.2 Complex III

This enzyme complex contains cytochromes \( b \) and \( c_1 \) and is referred to as ubiquinol-cytochrome \( c \) reductase (EC 1.10.2.2). It transfers electrons from reduced ubiquinone (ubiquinol) to cytochrome \( c \); this process is coupled to the pumping of protons from the matrix to the inner membrane space which sets up the proton gradient required for ATP synthesis. Complex III has a molecular mass of about 240 kDa and is made up of 11 subunits (Weiss et al. 1987).

1.1.3.3 Complex IV

This enzyme complex – Cytochrome \( c \) oxidase or Cox (EC 1.9.3.1) – is the terminal point in the ETC for the transfer of electrons from cytochrome \( c \), which it uses to reduce oxygen to water and to pump protons across the gradient for ATP
synthesis (Capaldi et al. 1982). Complex IV has 13 subunits and a molecular mass of about 210 kDa.

1.1.3.4 Complex II
The only enzyme complex that directly links the Krebs cycle to the ETC, Complex II, succinate-ubiquinone oxidoreductase (EC 1.3.5.1), oxidizes succinate to fumarate and transfers the electrons to ubiquinone. Unlike the other complexes, the Complex II proteins are all nuclear-encoded and together have a molecular mass of approximately 125 kDa.

1.1.3.5 Complex V and oxidative phosphorylation
The final steps in the respiratory chain involve the reduction of oxygen to water and the combination of ADP and inorganic phosphates to form ATP in the process called oxidative phosphorylation (OXPHOS) (Saraste 1999). There are three energy-conserving segments in the ETC as seen above (Complex I, Complex II + III and Complex IV) and each contributes to the production of one ATP molecule. The general equation for phosphorylating electron transport from NADH to oxygen is therefore:

\[
\text{NADH} + \text{H}^+ + \frac{1}{2} \text{O}_2 + 3\text{P}_1 + 3\text{ADP} \rightarrow \text{NAD}^+ + 3\text{ATP} + 4\text{H}_2\text{O}
\]

When electron pairs enter the ETC after Complex I, as is the case with some types of fatty acid and carbohydrate catabolism, only 2 molecules of ATP are produced by the respiratory chain.
The synthesis of ATP is catalysed by Complex V – F\textsubscript{0}F\textsubscript{1}-ATPase or ATP synthase (Penefsky \textit{et al.} 1960; Pullman \textit{et al.} 1960; Al-Awqati 1986). This complex has 16 subunits divided into 3 domains, the two major ones of which are the F\textsubscript{1} matrix globular domain and the F\textsubscript{0} transmembrane domain. The term F\textsubscript{0} comes from the capacity of this subunit to bind oligomycin, a potent inhibitor of ATP synthase activity (Kagawa and Racker 1966a, 1966b; Kagawa \textit{et al.} 1966). A chief postulate of the chemiosmotic coupling hypothesis is that ATP synthase can use the energy of ATP hydrolysis to pump protons across the inner membrane but since it can function reversibly if a large enough proton gradient is present, it can also allow protons to flow in the reverse direction to drive ATP synthesis (Thayer and Hinkle 1975a, 1975b).

The transport of ADP + phosphate from the cytosol to the mitochondrial matrix, and ATP in the reverse direction across the otherwise impermeable inner mitochondrial membrane, is facilitated by two types of highly specialised proteins known as translocases (Klingenberg 1979; Durand \textit{et al.} 1981). The first, the adenine nucleotide translocase, specifically allows the transport of ADP and ATP. It is a 32 kDa transmembrane protein and its dimers form a channel through which ADP or ATP is translocated across the membrane. The second is a phosphate-specific translocase, also a transmembrane protein.

The following graphic (\textbf{Fig. 1.4}) summarises the chemiosmotic flow of electrons along the ETC and the production of ATP in mitochondrial respiration.
Fig. 1.4 Flowchart of electron transport and ATP synthesis
1.2 Biogenesis of Mitochondria

1.2.1 Introduction

Although ATP was discovered in 1929 and its function established a few years later, there were two significant milestones in mitochondrial studies that occurred in the 1960s that paved the way for most modern discoveries in the field. The first was the proposal of the chemiosmotic coupling paradigm outlined above and the second was the discovery that all mitochondria contain a self-replicating genome that coded for some components of the ETC (Nass and Nass 1963a, 1963b; Borst 1972, 1977). With a couple of exceptions, all mitochondrial DNA (mtDNA) genomes are circular and double-stranded, and can vary in size from a few kilobases (kb) in animals to the massive 500+ kb mtDNA genomes in plants (Borst 1972, 1977; Attardi and Schatz 1988). While the genetic composition of these genomes is fairly constant, studies have shown considerable differences in their coding properties (Tzagoloff and Myers 1986). In general, mtDNA has been shown to contain two broad categories of genes – the “syn” genes that code for components of the mitochondrial protein synthetic machinery, and the “mit” genes that code for ETC proteins (Tzagoloff et al. 1979). A somatic mammalian cell can contain anywhere between 1000 and 10000 copies of mtDNA and mutations can be present in a percentage of these, a state known as heteroplasmy (Smeitink et al. 2001). Homoplasmy refers to the condition where all copies of mtDNA harbour a mutation or are wild-type.

The formation of mitochondria is a process as yet poorly understood but is known to involve both nuclear- and mtDNA-encoded products. As seen earlier,
mitochondria are highly membranous organelles. Since biological membranes arise from the growth and division of pre-existing membranes in a cell, the compartmentalisation of the mitochondrion is largely controlled by the nucleus. A fully-functional mitochondrion, as defined by its participation in ATP production through respiration, contains the components of the ETC described earlier, some of which are nuclear-encoded and the rest, mtDNA-encoded (Attardi and Schatz 1988). Studies have shown that cells lacking mtDNA can still respire anaerobically as well as show mitochondrial replication, and that repopulating these cells with mtDNA restores their respiratory chain activity, thus proving that mitochondriogenesis and function are controlled by both the nuclear and mitochondrial genomes (King and Attardi 1988, 1989).

The remainder of this chapter will focus on the human mitochondrial genome, unless otherwise mentioned.

1.2.2 Structure and Organisation of Human mtDNA

The human mitochondrial genome is one of the most compact eukaryotic genomes (Attardi and Schatz 1988) and comprises a 16,569 base-pair (bp) closed circular duplex DNA molecule whose sequence has been determined (Anderson et al. 1981). This sequence is referred to as the Cambridge Reference Sequence (CRS) and is available online, along with information on mtDNA polymorphisms, coding-region mutations and gene function (Ruiz-Pesini et al. 2007). It was subsequently corrected to account for nucleotide variations seen in different mtDNA samples as well as errors associated with the original sequencing
technique (Andrews et al. 1999). Like most mammalian mtDNA, its two strands – often called the “heavy” and “light” strands due to their differential densities in a cesium chloride gradient (Clayton et al. 1970; Berk and Clayton 1974) – code for two ribosomal RNAs (rRNA), all the transfer RNAs (tRNA) required for

Fig. 1.5 Map of human mtDNA. (The H-strand genes are shown on the outside and the L-strand genes on the inside. ND 1-6: NADH dehydrogenase, CO I-III: cytochrome c oxidase, ATP 6 & 8: H⁺-ATPase, cyt b: apocytochrome b. tRNA genes are represented by their corresponding 1-letter amino acid codes).
translation and a few of the components of the ETC (Anderson et al. 1981). Fig. 1.5 illustrates the general layout of human mtDNA.

There are no intronic sequences and most of the genes are “butt-jointed” or separated by only a few nucleotides (Eperon et al. 1980), and there are virtually no untranslated flanking regions in any open reading frame (Attardi and Schatz 1988). The origin of heavy-strand replication and the initiation sites for heavy and light-strand transcription occur in an area referred to as the “D-loop” or displacement loop, so called because of an intermediate triple-stranded replicon species (7S DNA) that contains a nascent ~680 bp fragment that loops out during replication (Anderson et al. 1981; Clayton 1991). This stretch of mtDNA, as well as the preceding ~500 bp before it in the closed circular genome, are fairly highly conserved among species and do not appear to possess any coding sequences.

The heavy strand codes for the 12S and 16S ribosomal RNAs, 14 tRNAs and 12 subunits of the respiratory chain. These are ND1, ND2, ND3, ND4, ND4L and ND5 (all subunits of NADH dehydrogenase), COI, COII and COIII (all subunits of cytochrome c oxidase), cytb (apocytochrome b), and, ATP6 and 8 (subunits 6 & 8 of ATP-synthase). The light strand codes for 8 tRNAs and subunit 6 of NADH dehydrogenase (ND6) (Anderson et al. 1981; Attardi and Schatz 1988).

Although many aspects of mitochondrial metabolism and macromolecular synthesis bear a close resemblance to similar processes in bacteria, one unique aspect of mtDNA is that it does not appear to code for 5S RNA. This is a ubiquitous ~120 base rRNA that plays a crucial role in translation in prokaryotic
and eukaryotic cells. It was initially suggested that 23 bases of a fragmentary 5S rRNA species were encoded at the distal end of the 16S rRNA sequence in human mtDNA (Nierlich 1982) but no further evidence has been presented to prove this. An RNA species with a size consistent with that of 5S RNA was found to be associated with purified human mitochondria (King and Attardi 1993) but its identity and mitochondrial origins could not be established. More recently, some evidence has been presented for the presence of a distinct 5S RNA species in mammalian mitochondria (Magalhaes et al. 1998), and it has been postulated that such a species is imported into the mitochondrion from the cytosol, although the mechanism by which this occurs is not understood.

The genetic code of human mtDNA (Table 1.1) differs from the universal genetic code in a few ways (Anderson et al. 1981). While the initiation codon AUG is retained, AUA and possibly AUU also function as such (Montoya et al. 1981). The normal termination codon UGA codes for tryptophan in mtDNA and AUA codes for methionine and not isoleucine as seen in nuclear DNA. Termination is driven by AGA and AGG codons, which normally code for arginine. Codons ending in A and C predominate while those ending in U or G are not used as frequently (Anderson et al. 1981).

One of the most striking features of the human mitochondrial genome is the way its sequence is arranged. Human heavy strand mtDNA is “punctuated” by tRNA genes – they are found on either side of the rRNA and mRNA coding sequences (Montoya et al. 1981; Ojala et al. 1981). The reason for this probably lies in the unique cloverleaf structure mature tRNAs assume – these structures are
recognition sites for cleavage of the transcribed genome. As will be shown below in a more detailed discussion of transcription, human mtDNA is transcribed into large polycistronic molecules that are precisely cleaved to yield mature RNA species. Any polyadenylation of the RNAs occurs post-transcriptionally and no part of the mtDNA sequence actually codes for this event (Clayton 1984, 1991).

| UUU | Phe | UCU | Ser | UAU | Tyr | UGU | Cys |
| UUC | Phe | UCC | Ser | UAC | Tyr | UGC | Cys |
| UUA | Leu | UCA | Ser | UAA | Ter | UGA | Trp |
| UUG | Leu | UCG | Ser | UAG | Ter | UGG | Trp |
| CUU | Leu | CUC | Pro | CAU | His | CGU | Arg |
| CUC | Leu | CCC | Pro | CAC | His | CGC | Arg |
| CUA | Leu | CCA | Pro | CAA | Gln | CGA | Arg |
| CUG | Leu | CCG | Pro | CAG | Gln | CGG | Arg |
| AUU | Ile/Met | ACU | Thr | AAU | Asn | AGU | Ser |
| AUG | Met | ACC | Thr | AAC | Asn | AGC | Ser |
| AUA | Met | ACA | Thr | AAA | Lys | AGA | Ter |
| AUG | Met | ACG | Thr | AAG | Lys | AGG | Ter |
| GUU | Val | GCU | Ala | GAU | Asp | GGU | Gly |
| GUC | Val | GCC | Ala | GAC | Asp | GGC | Gly |
| GUA | Val | GCA | Ala | GAA | Glu | GGA | Gly |
| GUG | Val | GCG | Ala | GAG | Glu | GGG | Gly |

Table 1.1 Mammalian mitochondrial genetic code
1.2.3 Replication of mtDNA

Like many molecular events in cell studies, the exact mechanism(s) of mtDNA synthesis have not been completely elucidated, although many basic components and enzymes involved in this process have been identified. Much of the early work in the area was carried out by Clayton and his co-workers and it was their “strand-displacement” model of mtDNA replication (Clayton 1982) that was widely accepted till a few years ago. However, Holt and colleagues proposed a “strand-coupled” mechanism (Holt et al. 2000; Yang et al. 2002; Bowmaker et al. 2003) that challenged the earlier hypothesis. To date, this is still a hotly debated topic and no apparent reconciliation between these two models exists (Brown et al. 2005; Falkenberg et al. 2007).

1.2.3.1 Initiation of mtDNA replication

As shown in Fig. 1.5 above, both the heavy and light strands of mtDNA have their own origins of replication (O_H and O_L respectively). O_H has been mapped to nucleotide position (nt) 191 in the D-loop region, while O_L has been shown to lie nested amongst a group of tRNAs for five amino acids, between nt 5730 and 5763 (Anderson et al. 1981). As noted above, the D-loop region contains the promoter for transcription of the light strand (LSP) and studies have shown that the RNA transcript originating from this site serves as the primer for the initiation of heavy strand synthesis (Clayton 1991). Sequence homology studies in vertebrate mtDNAs have shown the presence of three highly-conserved areas downstream of the LSP and in the D-loop (Walberg and Clayton 1983). These “Conserved Sequence Blocks” (CSBs I, II and III) might play a role in the stabilisation of this RNA-DNA hybrid intermediate replicon (Falkenberg et al. 2007). The ~30
nucleotides that comprise the origin of light strand replication are capable of forming a stable stem-loop structure (Clayton 1991) and while the exact relationship in light strand replication between this structure and the five tRNAs surrounding it are not known, it has been postulated that a RNA species binds to the loop and primes DNA synthesis near the stem (Clayton 1991). A DNA primase has been implicated in this process but has yet to be identified.

1.2.3.2 The strand-asymmetric (displacement) model of mtDNA replication
In this model, heavy (H) strand synthesis proceeds unidirectionally from the origin of replication (O_H). It displaces the H-strand as it proceeds and, as mentioned above, a triplex forms around 700 nt downstream of initiation – the so-called 7S DNA or D-loop, often terminating strand synthesis. The frequency of formation of this structure often depends on the physiological state of the cell and the precise reasons as to why and how often H-strand synthesis proceeds is not known. Experiments with a sea urchin transcription termination factor (mtDBP) have suggested the role of such a protein in the control of the progression of mtDNA replication (Fernandez-Silva et al. 2001). When it does proceed, the elongation of the H-strand continues until it reaches approximately two-thirds of the way around the genome. Here the strand displacement exposes the light (L) strand promoter (O_L), which in turn initiates elongation of the L-strand in the opposite direction. This asynchronous replication of the leading (H) and lagging (L) strands is what gives this widely-accepted mtDNA synthesis model its name, much of the evidence for which has come from the biochemical isolation and analysis of mtDNA replicative intermediates as well as electron microscopic studies of these species.
1.2.3.3 The strand-coupled model of mtDNA replication

As its name indicates, this model proposes that mtDNA synthesis is not asymmetric, but that the leading and lagging strands are in fact synthesised concurrently. Evidence for this has come from the use of neutral/neutral 2D agarose gel electrophoresis, in which DNA fragments are first separated by size and then by strand configuration in the second dimension. Replication intermediates with synchronous leading and lagging replication forks - forming so-called “y-arcs” - were visualised by this method. These y-arcs were present between the O_H and O_L, leading to the hypothesis that mtDNA replicates symmetrically, with multiple bidirectional replication forks. The initiation zone for bidirectional replication is thought to include the cytb and the ND5 and ND6 genes (Bowmaker et al. 2003).

The most recent findings by the proponents of both types of models do not appear to show any consensus. Atomic force microscopic studies seem to lend credence to the strand displacement theory, apart from predicting additional alternative origins of L-strand synthesis (Brown et al. 2005). This theory is also supported by the discovery of a major replication origin at nt 57 in the human mtDNA D-loop region (Fish et al. 2004). This origin has been described as being responsible for mtDNA synthesis under steady-state conditions, while the other D-loop origins may be responsible for mtDNA replication in response to physiological changes and mtDNA depletion. However, the parallel discovery of a bidirectional replication origin supports the strand-coupled model of mtDNA replication (Yasukawa et al. 2005). This origin maps to a region distal to the O_H, with
initiation occurring at nt 16184 on the L-strand and nt 16197 on the H-strand, both of which occur in the non-coding region (NCR) of mtDNA.

1.2.3.4 Factors associated with mtDNA synthesis

As noted above, many factors involved in mammalian mtDNA replication have been identified and characterised. These include mtDNA and mtRNA polymerases, mitochondrial single-stranded binding protein (mtSSB), mtDNA ligase, the Twinkle helicase and some topoisomerases (Lecrenier and Foury 2000; Moraes et al. 2002; Falkenberg et al. 2007).

The mtDNA polymerase γ was first identified as an RNA-dependent DNA polymerase in HeLa cells and consists of the catalytic α (or A) and the accessory β (or B) subunits. Subunit A is a 140 kDa protein that has polymerase, 3’-5’ exonuclease and 5’-deoxyribose phosphate lyase activities. Subunit B is a 55 kDa protein whose function has not been fully understood. It forms a heterodimer with Subunit A and can bind to double-stranded DNA stretches longer than 45 bp. It substantially increases the activity of Subunit A and possibly also promotes tighter substrate binding by the dimeric complex. mtDNA polymerase γ belongs to the family A group of DNA polymerases which includes the E.coli DNA pol I and the T7 DNA pol enzymes.

The mtSSB has an important role in mtDNA replication since the unwound strands of the DNA duplex need to be carefully manipulated during replication or repair. It is a 13-16 kDa protein which binds to single-stranded DNA as a tetramer and has homology to the E.coli SSB.
The TWINKLE gene was identified in patients with autosomal dominant progressive external ophthalmoplegia (adPEO), a disease in which multiple mtDNA deletions are seen and where the integrity of mtDNA was shown to be very low. Its gene product, the Twinkle protein, was later shown to be a novel mitochondrial protein with sequence homology to the T7 primase/helicase gene 4 protein (Spelbrink et al. 2001). Twinkle is therefore considered to be the replicative primase/helicase involved in mtDNA synthesis and maintenance and its preferred substrate has been shown to have the conformation of a DNA replication fork (Falkenberg et al. 2007).

1.2.4 Transcription of mtDNA

Early attempts to determine the nature of mtDNA transcription relied on the differential G+T content of the heavy and light strands (Clayton et al. 1970), since the complementarity of a given RNA species to either strand could be studied (Clayton 1984). Much of the initial work was done by Attardi and co-workers on HeLa cells and used radiolabeled RNA that hybridised to mtDNA (Aloni and Attardi 1971a, 1971b, 1971c) or electron microscopic examination of hybridised DNA-RNA products (Robberson et al. 1971; Wu et al. 1972; Murphy et al. 1975; Angerer et al. 1976). It was found that the H-strand encoded the largest number of stable transcripts and that rRNAs formed the most abundant species of mtDNA-encoded RNAs and they were the first mtRNAs to be precisely mapped (Crews and Attardi 1980; Eperon et al. 1980; Ojala and Attardi 1980; Ojala et al. 1980). These studies also showed that both strands of human mtDNA were symmetrically transcribed into almost genome-length polycistronic transcripts.
1.2.4.1 Initiation of mtDNA transcription

Transcription is normally initiated by the binding of a specialised enzyme called RNA polymerase to a specific sequence of DNA known as a promoter. Early experiments suggested that transcriptional initiation in mtDNA most likely began within the D-loop region (Cantatore and Attardi 1980). This concept was strengthened by direct evidence from mapping experiments that showed that the 5’ ends of both H- and L-strand transcripts- and hence the transcription initiation sites - mapped within the O\textsubscript{H} region (Montoya \textit{et al}. 1982). Plasmid deletion mutants were used to confirm this finding and showed that a repetitive AAACCCC sequence was found in the H-strand transcription start site as well as in initiation sites in other mammalian mitochondrial genomes (Bogenhagen \textit{et al}. 1984). Using an \textit{in vitro} system previously established by them (Walberg and Clayton 1983), Clayton and co-workers precisely mapped the promoters for both the H- and L-strands to a ~150bp locus inside the D-loop (Chang and Clayton 1984) and showed that they were functionally independent of each other. These promoters, named HSP (heavy strand promoter) and LSP (light strand promoter) and represented in Fig. 1.5 as H1 and LSP respectively, contain the 15bp motif 5’-CANACC(G)CC(A)AAAGAN-3’ which is necessary for effective transcription. Both promoters are approximately 50bp in length and also contain a sequence upstream to this motif that functions as a binding site for transcription factors (Chang and Clayton 1984; Hixson and Clayton 1985; Fisher \textit{et al}. 1987; Topper and Clayton 1989). They have also been shown to function bi-directionally (Chang D. D. \textit{et al}. 1986). H-strand transcription initiates at nt 561, next to the tRNA\textsubscript{Phe} gene, while L-strand transcription begins at nt 407 (Moraes \textit{et al}. 2002). The discovery that there were two overlapping H-strand transcripts (Montoya \textit{et al}. 2002).
(al. 1983) led to the conclusion that H-strand transcription was initiated at two sites. The second site (H2 in Fig. 1.5) is located before the start of the 12S rRNA gene at nt 638 (Moraes et al. 2002; Montoya et al. 2006) and its promoter region shows limited similarity with the 15bp consensus sequence seen in H1 and the LSP (Montoya et al. 1983). It also appears to be used less frequently for transcription of the H-strand (Moraes et al. 2002).

1.2.4.2 Mitochondrial transcription machinery

Mitochondria utilise a relatively simple system of enzymes and transcription factors that are unique to those found in the nucleus. It includes an RNA polymerase, at least three transcription factors, a termination factor and enzymes required for post-transcriptional processing.

The human mitochondrial RNA polymerase (POLRMT or mtRPOL) is a single subunit protein encoded by nuclear DNA (Tiranti et al. 1997). After its import into mitochondria, the 1230-amino acid precursor protein is cleaved of 41 residues at its amino terminal end, which generates the active 1189-residue POLRMT, whose N-terminal contains two 35-amino acid pentacotripeptide repeat (PPR) motifs (Rodeheffer et al. 2001) that have been implicated in RNA processing in mitochondria and chloroplasts (Mili and Piñol-Roma 2003). It is currently not known if the PPR motif is an RNA-binding domain and its exact role in mammalian mitochondrial transcription remains to be established. The C-terminal of POLRMT, from residue 520 to residue 1230, contains a series of conserved motifs also found in bacteriophage polymerases (Masters et al. 1987). POLRMT cannot interact with promoter DNA or initiate transcription on its own, and
requires one or more transcription factors for its specific activity (Fisher and Clayton 1985; Prieto-Martin et al. 2001).

Mitochondrial transcription factor A (mtTFA or TFAM) is a 25 kDa protein that was first isolated from human mitochondria (Fisher and Clayton 1985) and shown to bind sequences upstream of the transcription start sites on the H- and L-strands (Fisher et al. 1987). The TFAM binding sites on both strands show significant homology but studies have shown that it binds with greater affinity to the LSP (Ghivizzani et al. 1984; Fisher et al. 1987; Fisher and Clayton 1988). The 2469 residue pre-protein is nuclear-encoded and is cleaved into an active 202-residue form after import into the mitochondrion. It contains two high mobility group (HMG) domains involved in DNA-binding, separated by a 27-residue linker and followed by a 25-residue basic C-terminal tail (Fisher and Clayton 1988; Parisi and Clayton 1991). HMG domains have also been implicated in transcription enhancement and chromatin packaging (Moraes et al. 2002). Mutation analysis has shown that the C-terminal tail is crucial for specific DNA recognition and is also essential for efficient transcription (Dairaghi et al. 1995a). Like other proteins of the HMG domain family, TFAM can bind to, bend and unwind DNA in a non-sequence specific manner and this ability has been implicated in its stimulation of mtDNA transcription (Fisher et al. 1992), especially in introducing specific structural alterations in mtDNA to facilitate POLRMT binding and transcription initiation. Although the HSP and LSP function in both directions, the asymmetric binding of TFAM to them, relative to their transcription start sites, might ensure that transcription is unidirectional. Some evidence for this came from a study that showed that the 10 bp spacing between the TFAM binding site and the
transcription start site on the LSP was essential for efficient L-strand transcription (Dairaghi et al. 1995b). These L-strand transcripts also function as primers for mtDNA replication (Montoya et al. 1997; Gensler et al. 2001), implicating TFAM as an important factor in this process.

Using the bioinformatics alignment tool PSI-BLAST, two additional mitochondrial transcription factors, TFB1M and TFB2M, have been identified (Falkenberg et al. 2002; McCulloch et al. 2002). Both display sequence similarity to a family of rRNA methyltransferases, which dimethylate two adjacent adenosine bases near the 3’ end of the small subunit rRNA during ribosome biogenesis. TFB2M might be a specialised transcription factor in mammals (Rantanen et al. 2003) and shows at least two orders of magnitude more activity in stimulating specific transcription than TFB1M (Falkenberg et al. 2002). Both factors form heterodimeric complexes with POLRMT and either one, in addition to TFAM, is required for accurate transcription initiation in vitro of H1 and LSP, but do not appear to be necessary for transcript elongation (Falkenberg et al. 2002). TFB1M has also been shown to function as an rRNA methyltransferase (Seidel-Rogol et al. 2003), but its transcription and methylation activities appear to be independent of each other (McCulloch and Shadel 2003).

1.2.4.3 Elongation and termination of mtRNA transcripts

The three mitochondrial transcription units starting from H1, H2 for the H-strand and the LSP for the light strand all produce polycistronic transcripts that are later processed into mature RNA species. The L-strand is transcribed as a single polycistronic precursor RNA that contains most of the genetic information.
encoded by it (Murphy et al. 1975; Montoya et al. 1981) and termination of its transcription has not been studied in detail. There is some evidence from murine mtDNA studies that this event occurs just immediately upstream of the tRNA^{Phe} gene and that two proteins with sizes of 45kDa and 70kDa are involved (Camasamudram et al. 2003).

The transcription of the H-strand is unique in that there are two overlapping polycistrons that are produced (Montoya et al. 1983); the H1 transcript contains the tRNAs for phenylalanine and valine, and the two rRNAs, while the H2 transcript is genome length. This led to the conclusion that a termination event downstream of the 16S rRNA gene was responsible for the abbreviated H1 transcript and, along with the finding that the H2 transcript is not produced as much as the H1 transcript (Montoya et al. 1982), was thought to explain the finding by Gelfand and Attardi (1981) that in all studied cells, the rRNAs are synthesised at a much higher rate than the individual mRNAs. In vitro assays showed that a ~ 11-12 bp sequence at the 16S rRNA-tRNA^{Leu} boundary appeared to drive transcription termination and that this sequence could function in both directions (Christianson and Clayton 1986). Further studies showed that the tridecamer sequence 5’-TGGCAGAGCCCGG-3’ contained within the tRNA^{Leu} gene was necessary for accurate transcription termination (Christianson and Clayton 1988).

Subsequently, a ~39kDa protein was isolated that bound to a 28 bp region spanning this sequence and shown to be responsible for H1 transcription termination (Kruse et al. 1989; Daga et al. 1993). It was named mTERF.
(mitochondrial transcription termination factor) and is nuclear-encoded. It contains three potential leucine zippers and two distinct basic binding domains and binds to mtDNA as a monomer. It is thought that the leucine zippers interact intramolecularly, rather than with the DNA, to bring the basic domains together for DNA–binding (Fernandez-Silva et al. 1997). mTERF exists in two forms in mitochondria, an active monomeric form and as an inactive homotrimer (Asin-Cayuela et al. 2004). A rearrangement of the leucine zippers is thought to be responsible for the switch between the two forms, with intermolecular zipper interactions causing trimerisation and inactivity. mTERF also does not appear to require phosphorylation in order to gain activity (Asin-Cayuela et al. 2005) although there is some evidence of this in other species (Montoya et al. 2006). However, its termination activity is polarity-specific with respect to the orientation of its binding site; there is a significant loss of activity when the mTERF recognition sequence is reversed relative to the H1 promoter (Asin-Cayuela et al. 2005). mTERF preferentially binds a similar conserved sequence on the L-strand which might explain its orientation-dependent activity (Nam and Kang 2005). It has been suggested that mTERF may also stop L-strand transcription at a binding site where no genes are present downstream (Hess et al. 1991), which is supported by the finding that it promotes termination of transcription by heterologous RNA polymerases only in the L-strand direction of transcription (Falkenberg et al. 2007). Recombinant human mTERF completely blocks L-strand transcription in an in vitro system but is not as effective at terminating H-strand transcription (Asin-Cayuela et al. 2005). A possible mode of action of mTERF is to create a physical barrier to POLRMT, rather than to interact with it and prevent its functioning (Shang and Clayton 1994).
Interestingly, the discovery that a mutation in the mTERF binding site only reduces its affinity to bind mtDNA but not the ratio of H1 and H2 transcripts suggests that mTERF’s primary role might not be in transcription termination (Chomyn et al. 1992). The earlier proposals that this protein may also be involved in transcription initiation (Kruse et al. 1989; Asin-Cayuela et al. 2004) were strengthened by the discovery of a second binding site for mTERF at the H1 locus (Martin M. et al. 2005). Electron microscopic studies have shown that mTERF creates a loop between H1 and the end of the 16S rRNA gene. This implies that it controls the expression of the H1 transcript and may facilitate the reinitiation of transcription (Martin et al. 2005; Falkenberg et al. 2007).

Fig. 1.6 Human mtDNA transcription (The dotted arrows represent the two major classes of H-strand transcripts that are produced).
The complexity underpinning the mechanism of mtDNA transcription and its termination has been increased by recent bioinformatics analyses that have identified three novel genes in vertebrates that code for proteins homologous to mTERF, all of which have predicted mitochondrial localisation (Linder et al. 2005). They have been named mTERF2-4, with mTERF1 being the new name for mTERF. The mitochondrial localisation of mTERF2 has been confirmed in humans and gene expression analysis has shown high expression in heart, liver and skeletal muscle, a pattern typical of mitochondrial proteins (Chen et al. 2005). However, its exact function remains to be elucidated. Fig. 1.6 schematically illustrates the human mitochondrial transcription control and termination regions.

1.2.4.4 Post-transcriptional modifications

Human mtDNA does not contain any introns and intergenetic sequences are minimal. The H1 transcript codes for two tRNAs and the two rRNAs, while the H2 transcript codes for 14 tRNAs and 12 OXPHOS polypeptides. The L-strand transcript gives rise to 8 tRNAs and ND6 mRNA. These polycistronic transcripts are processed according to the “tRNA punctuation model” mentioned earlier (Ojala et al. 1980; Ojala et al. 1981). According to this model, the tRNA sequences on the nascent transcripts fold into their mature cloverleaf structures, which act as signals for enzymes that cleave the precursor RNA on either side of the tRNAs, giving rise to the various mature RNA species. All the enzymes involved in post-transcriptional processing of mtRNA are nuclear-encoded (Clayton 1991).
The processing of the polycistronic transcripts requires precise cleavage at the 5’ and 3’ ends of the tRNA sequences. The 5’-endonucleolytic cleavage occurs first (Rossmanith et al. 1995) and is accomplished by a mitochondrial RNase known as mtRNase P (Doersen et al. 1985; Puranam and Attardi 2001). The 3’-end cleavage is performed by tRNase Z (Levinger et al. 2001). tRNA molecules normally have a conserved CCA sequence at their 3’ ends that regulates the attachment of their corresponding amino acids at that end. The addition of the CCA tail to mitochondrial tRNAs is catalysed by an ATP(CTP):tRNA nucleotidyltransferase (Nagaike et al. 2001). The CCA tail also prevents its own removal by tRNase Z. While the precise mechanism is not known, it has been postulated that post-transcriptional degradation events might play a role in maintaining the steady state of mitochondrial tRNAs (King and Attardi 1993).

Polyadenylation of mitochondrial mRNAs and rRNAs is performed by a mitochondrial poly(A)-polymerase (Tomecki et al. 2004). mRNAs are polyadenylated with tails of about 55 A residues (Ojala and Attardi 1974) while rRNAs from the H1 transcript are either not polyadenylated or have very short poly(A) tails added (Dubin et al. 1982). Although the H2 rRNA transcripts are polyadenylated (Montoya et al. 1983), they are considered waste products which do not lead to ribosome formation (Dubin et al. 1982). Unlike in bacteria, the poly(A) tails appear to increase the stability of the RNAs (Nagaike et al. 2005) and in many cases produces the stop codons for mRNA translation (Ojala et al. 1981).
1.2.5 Translation of mitochondrial transcripts

Very little information is available on the molecular events in mitochondrial translation. Studies on rat liver mitochondria have shown that there are close to a 100 ribosomes per mitochondrion (Cantatore et al. 1987). Mammalian mitochondrial ribosomes differ in many aspects from cytosolic and prokaryotic ones and have an unusually low RNA content (Hamilton and O'Brien 1974). 2D gel electrophoresis has identified 85-86 spots from mammalian ribosomes and some of these proteins might have adopted structural and functional roles of rRNA sequences (Moraes et al. 2002).

Since mammalian mitochondrial mRNAs have no 5’ untranslated region (UTR) or cap structure, coding sequences begin very close to the codon for the translation initiating N-formylmethionine (Montoya et al. 1981). Studies have shown that around 400 nucleotides are required for efficient binding of the small ribosome subunit but a smaller region actually interacts with the ribosome (Denslow et al. 1989; Liao and Spremulli 1989, 1990). This binding is followed by the movement of the ribosome subunit to the 5’ end of the mRNA, an event possibly facilitated by factors as yet uncharacterised. The only mammalian translational initiation factor identified thus far is mtIF-2, a monomeric GTPase protein (Liao and Spremulli 1991).

Translation elongation factors were first identified in bovine liver mitochondria and named mtEF-Tu, mtEF-Ts and mtEF-G (Schwartzbach and Spremulli 1989; Chung and Spremulli 1990). The human cDNAs for all three have been cloned and sequenced and in vitro analyses suggest that they function in a similar manner
to prokaryotic elongation factors (Ma and Spremulli 1995; Worix et al. 1995; Xin et al. 1995).

Release factors enable the translated protein to detach from the ribosomal machinery and the cognate mRNA. Until recently, the only human candidate protein – mtRF1 -was proposed by largely bioinformatics analyses (Zhang and Spremulli 1998) but a new study appears to have isolated the first human protein – mtRF1a - with mitochondrial localisation and release factor-like functions (Soleimanpour-Lichaei et al. 2007).

1.2.6 Protein import into mitochondria

The human mitochondrion encodes 13 proteins, which is less than 1% of the mitochondrial proteome thought to be involved in the biogenesis of mitochondria (Mokranjac and Neupert 2005). Of the approximately 1500 proteins involved in this process (Mootha et al. 2003), over 600 have been identified using a variety of genetic and molecular approaches (Mootha et al. 2003; Ozawa et al. 2003; Taylor et al. 2003). All of these are nuclear-encoded and synthesised in the cytosol as precursors, which are then targeted to the mitochondrion and translocated into it by a highly-conserved mechanism in eukaryotes (Neupert 1997; Wiedemann et al. 2004). Cytosolic chaperones maintain these precursor proteins, which contain targeting sequences, in a translocation-competent conformation (Neupert and Herrmann 2007).

Mitochondrial precursor proteins can be divided into two main classes – those that carry N-terminal cleavable extensions or “presequences”, as seen above with
transcription factors, and those that contain internal targeting signals. The N-terminal targeting sequences are also called Matrix Targeting Sequences or MTSs and preproteins that contain them are destined for the matrix, the inner membrane or the intermembrane space of the mitochondrion (Neupert and Herrmann 2007).

The other class of preproteins generally targets the outer membrane, though some are destined for the inner membrane (Wiedemann et al. 2004). Presequences are positively charged sequences of 10-80 amino acids that interact with import receptors on the outer and inner membranes (Schatz and Dobberstein 1996). Preproteins with internal targeting sequences typically have a different conformation from the corresponding mature active proteins (Koehler et al. 1999).

**Fig. 1.7 Illustration of the various protein import pathways into mitochondria**

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The translocase of the outer mitochondrial membrane (TOM complex) is the primary entry gate for nuclear-encoded preproteins. It consists of seven subunits that are grouped into 3 categories – the receptors Tom20, Tom22 and Tom70, the β-barrel channel protein Tom40, and the structural small proteins Tom5, Tom6 and Tom7 (Hill et al. 1998; Kunkele et al. 1998; van Wilpe et al. 1999).

According to the “binding chain hypothesis” (Komiya et al. 1998; Meisinger et al. 2001), the MTS of a nuclear preprotein first binds to Tom20 and with the help of Tom5, is transported to the import pore formed by Tom40. After translocation through Tom40, the presequence binds to the intermembrane domain of Tom22. Tom6 and Tom7 are involved in the assembly and stability of the TOM complex (Model et al. 2001). Preproteins with internal signal sequences are recognized by Tom70 which interacts with their molecular chaperones, the heat shock proteins Hsp70 and Hsp90, before delivering them to Tom40 (Neupert and Herrmann 2007).

After passing through the TOM complex, precursor proteins generally follow one of three pathways (Wiedemann et al. 2004). The first of these involves the translocase of the inner membrane – the TIM23 complex - and the molecular import motor proteins mtHsp70 (matrix heat shock protein), Tim44, Tim14 and Tim16, which comprise the presequence translocase associated motor or PAM. The TIM23 complex consists of three integral membrane proteins, Tim50, Tim23 and Tim17 (Jensen and Johnson 2001; Pfanner and Geissler 2001) and a fourth newly-identified subunit, Tim21 (Chacinska et al. 2005). Presequence carrying proteins first bind to Tim50 which guides them to the import pore formed by Tim23, a process dependent on the membrane potential across the inner
mitochondrial membrane. The exact function of Tim17 and Tim21 are unclear. Since the membrane sector of the TIM23 complex can only transfer the MTS of preproteins, the import motor (PAM) then takes over and completes the translocation of the complete polypeptide chain.

The second pathway is used by precursor proteins of the hydrophobic inner membrane proteins which do not contain MTSs. They utilise the carrier translocase protein insertion machinery of the TIM22 complex and various chaperone-like components of the intermembrane space (IMS), notably the small proteins Tim9 and Tim10 (Wiedemann et al. 2004). The Tim22 complex is a 300 kDa multimer consisting of Tim22, Tim54 and Tim18 (Neupert and Herrmann 2007). Preproteins are transferred to the import pore Tim22 by the Tim9-Tim10 complex and the translocation across the Tim22 channel is also mediated by the inner membrane potential (Sirrenberg et al. 1996; Kovermann et al. 2002). The precise functions of Tim54 and Tim18 are unknown (Neupert and Herrmann 2007).

Outer membrane proteins which have a complex topology such as multiple β-barrel strands or with several transmembrane segments (such as porin) utilise a third pathway to reach their destination within the mitochondrion. Their precursors are first imported by the TOM complex to the IMS, where, with the help of the small TIM proteins, they are passed onto the Sorting and Assembly Machinery (SAM) complex. This was identified by analysing the outer membrane protein Mas37 (Wiedemann et al. 2003) and one of its components is the highly
conserved essential outer membrane protein Sam50 also known as Tob55/Omp85 (Kozjak et al. 2003; Paschen et al. 2003; Gentle et al. 2004).

Intermembrane space proteins are generally classified on the basis of their import characteristics into one of three types: bipartite proteins that have an N-terminal MTS and a hydrophobic sorting sequence, proteins that utilise a cofactor to fold correctly and thereby avoid escape from the IMS, and proteins that are permanently associated with factors in the IMS (Neupert and Herrmann 2007).

The biogenesis of mitochondria therefore involves the growth and division of pre-existing organelles, with mtDNA coding for a small fraction of the components required for this process. The presence of this unique separate genome in the organelle has led to much speculation about its origins and the reasons for its existence in a modern eukaryotic cell.

1.2.7 Origin and evolution of mitochondria and mtDNA

The emergence of mitochondria is considered a defining event in the evolution of eukaryotic cells. The most favoured model for explaining this event is the “serial endosymbiosis theory” which postulates that mitochondria are the direct descendants of a bacterial endosymbiont that became established in a nucleus-containing but amitochondriate host cell (Gray et al. 1999). Analysis of mtDNA sequence and expression data has shown a striking similarity to these features in eubacteria (Gray and Doolittle 1982; Gray 1989) and phylogenetic analysis of mtDNA indicates that mitochondria evolved from a single ancestor in the α-group of Protobacteria (Yang et al. 1985). *Rickettsia prowazekii*, the causative agent of
epidemic louse-borne typhoid, is a member of this sub-group and its genome sequence (Andersson et al. 1998) shows that it is the closest relative of the mitochondrion.

Although there is compelling evidence for the monophyletic origin of mitochondrial evolution, some studies have questioned the serial endosymbiont hypothesis. The “hydrogen hypothesis” (Martin and Muller 1998) claims that an association between a hydrogen-producing protobacterium and a hydrogen-requiring archaeabacterium could have led to the simultaneous evolution of both the nucleus and mitochondrion in a eukaryotic cell.

A large number of mitochondrial genomes have been completely sequenced and sequence analyses of these using databases such as GOBASE (Korab-Laskowska et al. 1998) has led to the conclusion that mtDNAs come in two basic types, ancestral and derived (Gray et al. 1999). Ancestral mitochondrial genomes show clear homology to eubacterial ancestors and are characterised by:

(i) the presence of many extra genes such as additional NAD genes

(ii) eubacteria-like 16S, 23S and 5S rRNAs

(iii) a complete set of tRNA genes

(iv) few intronic sequences and eubacterial gene clusters, and,

(v) a standard genetic code.

Derived mtDNAs retain little of their ancestral traits and are characterised by a reduction in genome size, such as is seen in human mtDNA. The evolution of these genomes is marked by:
(i) extensive gene loss
(ii) a marked divergence in rDNA and rRNA structure
(iii) accelerated mutation rates, and,
(iv) changes in codon usage and use of a non-standard genetic code.

Mammalian mtDNA rapidly evolves (Cantatore and Saccone 1987) and undergoes sequence divergence at a 5-10-fold higher rate than nuclear DNA (Brown et al. 1979; Miyata et al. 1982). Sequence changes include >90% silent substitutions, ~10% replacement substitutions and a strong bias towards C to T transitions in the L-strand. The rates of sequence divergence decrease in the order D-loop > protein-coding genes > rRNA and tRNA genes (Gray 1989). Twelve of the thirteen protein-coding genes of human mtDNA show their own characteristic rates of change; the CoxII gene has shown a 5-fold acceleration in its rate of divergence which is coupled to a parallel acceleration in the rate of change of the nuclear-encoded cytochrome c protein (Gray 1989). tRNA genes change in sequence at about one-half the rate of protein-coding genes but still evolve 100 times faster than nuclear tRNA genes, possible due to more relaxed functional constraints (Brown et al. 1982).

The high substitution rate, the apparent lack of recombination in mtDNA (Olivo et al. 1983) and the report that mtDNA is maternally inherited (Giles et al. 1980), led researchers to analyse human evolution from a matrilineal (mitochondrial) viewpoint. Early studies, mostly restricted to D-loop (control region) sequence comparisons between mtDNA samples, showed that all modern humans probably descended from a common female ancestor in Africa (Cann et al. 1987; Vigilant
et al. 1991), the so-called “Mitochondrial Eve” hypothesis. Subsequent analysis of complete mtDNA sequences confirmed these findings (Ingman et al. 2000) and human mtDNA samples were classified into “haplogroups” on the basis of their polymorphisms and continental origins. There are nine major European, three major African and five major Asian haplogroups, each denoted by a unique letter of the alphabet (Herrnstadt et al. 2002) and several of these are further divided into sub-haplogroups.

The dynamics of mitochondrial evolution and inheritance, as well as the large number of mtDNA polymorphisms, play a critical role in human mitochondrial disorders localized to cells with high energy demands such as neurons and muscles, in which mitochondria vastly over-proliferate relative to healthy cells. The next section will discuss mitochondrial mutations and their effect on the metabolism of these cells.
1.3 Mitochondrial Dysfunction

1.3.1 Introduction

The term “mitochondrial disorders” usually refers to diseases which are marked by abnormalities in OXPHOS metabolism. Mitochondrial defects occur in a wide variety of degenerative diseases, aging and cancer (Wallace 1999). The earliest indication that mitochondria play a role in pathogenesis came from a report of a patient with hypermetabolism whose skeletal muscle contained large numbers of abnormal mitochondria (Luft et al. 1962). This condition, now known as mitochondrial myopathy, involved “loose coupling”, a defect in the coupling between respiration and oxidative phosphorylation. Since then, mitochondrial dysfunction has been implicated in a wide variety of clinical problems, especially in disorders of the CNS, whose cells require large amounts of ATP for proper function (Kann and Kovacs 2007). A frequently seen mitochondrial OXPHOS disorder is Leigh syndrome, a progressive subcortical encephalopathy (Leigh 1951; Rahman et al. 1996).

The molecular basis of mitochondrial diseases is very complex and not completely understood. They can be caused by changes in mtDNA, in the form of mutations, insertions or deletions (Wallace 1992; Larsson and Clayton 1995). mtDNA diseases commonly have a delayed onset and a progressive course, which implies that aging exacerbates the genetic defect (Wallace 1999). The first such disease to be understood at the molecular level was Leber’s hereditary optic neuropathy (LHON), a maternally-inherited sudden onset blindness resulting from an mtDNA missense mutation (Wallace et al. 1988). Deletions in mtDNA were shown to play
a role in the pathogenesis of the neuromuscular diseases CPEO (chronic progressive external ophthalmoplegia) and KSS (Kearns-Sayre syndrome) (Holt et al. 1988). Since then, hundreds of such mtDNA mutations have been implicated in the pathogenesis of human diseases (Ruiz-Pesini et al. 2007). Some mitochondrial diseases have also been linked to nuclear genes, whose inactivation either inhibit mitochondrial function or disrupt mtDNA biogenesis (Wallace 1999).

Mitochondrial diseases can have a wide variety of inheritance patterns – maternal, Mendelian or a combination of the two (Wallace 1999). The important differences between mitochondrial genetics and Mendelian genetics that play a role in OXPHOS diseases are (Wallace 1992; Smeitink et al. 2001):

(i) the more or less strict maternal inheritance of mtDNA
(ii) the interplay of nuclear and mitochondrial genes in biogenesis
(iii) polyplasmy and heteroplasmy of mtDNA
(iv) the threshold effect, where a critical number of mutated mtDNAs must be present for OXPHOS malfunction, and,
(v) the high rate of mutation in mtDNAs.

Another complicating factor is the fact that the phenotypes of mitochondrial diseases can be both diverse and overlapping – the same mtDNA mutation can produce different phenotypes and different mutations can produce the same phenotype. In general, mitochondrial disorders are classified by genetic defect rather than by clinical manifestation (Wallace 1999).
1.3.2 mtDNA mutations and disease

There are several types of mtDNA mutations that have been implicated in the pathogenesis of human diseases. They can take the form of missense mutations, insertions, deletions, protein synthesis (biogenesis) mutations and copy number mutations.

1.3.2.1 Missense mutations

These involve changes in the mtDNA coding sequence that cause the corresponding amino acid to be substituted and have been associated with LHON and NARP (Neurogenic muscle weakness, Ataxia and Retinitis pigmentosa). LHON is caused by a number of mtDNA mutations but ~70% of the time, it is associated with a G-A substitution at nt 11778. This changes the 340th amino acid of ND4 from an arginine to a histidine (Wallace et al. 1988). In large pedigrees, this mutation is essentially homoplasmic. In ~13% of LHON cases, a G-A transition is seen at nt 3460, causing an alanine to threonine substitution in the 52nd amino acid residue of ND1 (Howell et al. 1991a; Howell et al. 1991b). About 14% of LHON cases are caused by a T-C change at nt 14484, which changes the 64th amino acid of ND6 from methionine to valine (Brown M. D. et al. 1992; Johns et al. 1992). Some rarer LHON missense mutations include G3733A, C4171A, T10663C, G14459A, C14482G, C14482A, A14495G and C14568T (Ruiz-Pesini et al. 2007).

NARP is largely caused by the T-G transversion at nt 8993, which changes amino acid 156 of Atpase6 from leucine to arginine (Holt I. J. et al. 1990). The disease
is characterised by heteroplasm, with severity being related to the percentage of mutant mtDNA in the patient.

1.3.2.2 Deletions and insertions in mtDNA

Single deletions of mtDNA, typically seen in mitochondrial myopathy, CPEO and KSS, are large, encompass several tRNA and protein-coding genes and each patient normally has only one type of deletion in a heteroplasmic mixture with wild-type mtDNA (Holt et al. 1988). Such deletions usually occur spontaneously and maternal inheritance is rare (Larsson et al. 1992). Deleted mtDNA levels are higher in affected tissues and can cause respiratory chain malfunction (Hayashi et al. 1991). Children generally show a more widespread tissue distribution of deleted mtDNA than adults, and earlier onset of disorders that affect multiple organs (Larsson and Clayton 1995). Infants with deleted mtDNA develop Pearson’s syndrome, a generally fatal disorder (Rötig et al. 1990).

A large number of single deletions of mtDNA have been described and range in size from 1-10 kb (Ruiz-Pesini et al. 2007). Approximately 30-50% of patients harbor a common 4997 bp deletion between nt 8468 and nt 13446, both of which are flanked by a 13 bp (5’-ACCTCCCTCACCA-3’) repeat that represents a hotspot for the formation of deletions in human mtDNA (Schon et al. 1989; Shoffner et al. 1989). Deletions that are flanked by such short direct repeats are termed Class I deletions while those in Class II have no (or imperfect) repeats flanking them (Larsson and Clayton 1995). To date, no deletions that remove O_H, O_L or the LSP have been described. Deletions affecting the HSP have been shown
in a few patients; these deletions prevent H-strand transcription but not L-strand transcription or mtDNA replication (Moraes et al. 1991).

The molecular basis for the formation of single deletions has not been elucidated but mechanisms including homologous recombination (Schon et al. 1989) and slip-replication (Shoffner et al. 1989) have been proposed. The latter theory might account for some mtDNA deletions, since DNA breaks have been shown to occur a few nucleotides from the direct repeat. This causes the downstream repeat to be lost and base-pairing of the two remaining repeats on the two strands during replication, eliminating the mtDNA in between them.

Multiple mtDNA deletions have been reported in many phenotypes, including neurogastrointestinal encephalomyopathy, inclusion body myositis and autosomal dominant PEO (Larsson and Clayton 1995; Ruiz-Pesini et al. 2007). In many cases, the levels of deleted mtDNA were far below the threshold required to cause a respiratory chain deficiency.

Complete mtDNA duplication was first reported in a case of leukemia (Clayton and Vinograd 1967) and took the form of unicircular dimers, with the two molecules joined head to tail. These dimers are present only in tumour tissue and are therefore somatic mtDNA mutations. Partial duplications of mtDNA was first found in two patients with ocular myopathy and diabetes mellitus (Poulton et al. 1989a, 1989b). The duplications extended from the COII region to the cytb region and included the O_H and O_L. Partial mtDNA duplications are always present in a heteroplasmic mixture with wild-type mtDNA and have been shown to be
maternally inherited (Rötig et al. 1992). The mechanism of formation of mtDNA duplications is not known but it has been suggested that it might be similar to the ones responsible for the formation of deletions.

Deletions and duplications have also been reported in the non-coding regions of human mtDNA. In vitro studies have shown that heteroplasmy with length heterogeneity in the CSB II sequence is present in human cultured cells and that transcription was not affected (Hauswirth and Clayton 1985). A heteroplasmic duplication of ~270 bp involving the HSP, LSP, CSB II and CSB III was initially described in patients with single mtDNA deletions (Brockington et al. 1993) but later shown to be a possible polymorphism in normal Caucasians (Torroni et al. 1994). Studies with other animal mtDNAs have also shown heterogeneity within the D-loop, indicating that it is a mutational hot spot for mtDNA re-arrangements.

1.3.2.3 Biogenesis mutations

Nucleotide substitutions in the mitochondrial biosynthetic rRNA and tRNA genes generally have more systemic phenotypic consequences than missense or other mutations (Wallace 1992). The earliest mutations identified in the 16S rRNA of mammalian mitochondria were point mutations at its 3’ end that conferred resistance to chloramphenicol, a drug that selectively inhibits mitochondrial protein synthesis (Kearsey and Craig 1981). A G3196A substitution was found in patients with ADPD (Alzheimer’s Disease and Parkinson’s Disease) (Shoffner et al. 1993). A C3093G mutation was reported in a patient with MELAS (Mitochondrial Encephalomyopathy, Lactic Acidosis and Stroke-like symptoms), diabetes mellitus, hyperthyroidism and cardiomyopathy (Hsieh et al. 2001). Some
studies have linked a C2853T substitution to Rett syndrome (Ruiz-Pesini et al. 2007).

A point mutation in the 12S rRNA (A1555G) has been observed in a large family with nonsyndromic congenital deafness (Prezant et al. 1993) and in families with aminoglycoside-induced deafness (Bacino et al. 1995). Several other mutations in the region have also been linked to deafness, including the A827G, T961C, T1005C, A1116G and C1494T substitutions (Ruiz-Pesini et al. 2007). A T1095C point mutation was described in a pedigree with sensorineural hearing loss, parkinsonism and neuropathy (Thyagarajan et al. 2000). Two other mutations, C1310T and A1438G, have been linked to diabetes mellitus.

Several tRNA mutations have been reported that are associated with mitochondrial myopathy. In severe cases, several organ systems may be involved in disease progression (Wallace 1992). Among the diseases linked to tRNA mutations are MELAS, MERRF (Myoclonic Epilepsy and Ragged-Red Fibre Disease), MMC (Maternally Inherited Myopathy and Cardiomyopathy), CPEO, LIMM (Lethal Infantile Mitochondrial Myopathy), Ocular myopathy, ADPD, Multiple sclerosis, Diabetes mellitus and deafness (Ruiz-Pesini et al. 2007). Recently, a tRNA\textsuperscript{Ile} T4274C mutation was described in a patient with Motor Neuron Disease (MND) (Borthwick et al. 2006). Two of the most important mitochondrial tRNAs implicated in human disease are the tRNA\textsuperscript{Lys} and the tRNA\textsuperscript{Leu(UUR)}. 

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Mutations in tRNA$^{\text{Lys}}$ are responsible for MERRF, a maternally inherited disease in which patients have uncontrollable myoclonic epilepsy. A majority of the patients with MERRF harbour a heteroplasmic A-G transition at nt 8344, which alters a highly conserved base of the TΨC loop of tRNA$^{\text{Lys}}$ (Shoffner et al. 1990). A less frequent T8356C mutation has also been reported in patients with MERRF and disrupts a highly conserved base in the TΨC stem of tRNA$^{\text{Lys}}$ (Silvestri et al. 1992). Cell studies have shown that these mutations inhibit mitochondrial protein synthesis and cause a respiratory chain deficiency (Chomyn et al. 1991; Masucci et al. 1995). The A8344G mutation has also been shown to cause defective aminoacylation of tRNA$^{\text{Lys}}$ and premature termination of mitochondrial translation (Enriquez et al. 1995). The severity of MERRF is directly proportional to the mutant load in patients and MERRF pedigrees have reduced Complex I and IV activities.

The tRNA$^{\text{Leu(UUR)}}$ gene has been described as an etiologic hotspot for pathogenic mitochondrial mutations (MoraesT. et al. 1993a). Eleven important mutations have been described in this region, several of which are associated with MELAS. Fig. 1.8 illustrates the different locations of disease-causing mutations in the tRNA$^{\text{Leu(UUR)}}$ gene.
About 80% of MELAS cases, 20% of PEO cases and a small number of diabetes patients have a A3243G substitution in their tRNA\textsubscript{Leu(UUR)} gene (Goto \textit{et al.} 1990; Reardon \textit{et al.} 1992; Moraes \textit{et al.} 1993b). This mutation alters the dihydrouridine loop of the tRNA\textsubscript{Leu} and inactivates the binding of mTERF to its recognition sequence present in the region. Although cellular studies in MELAS patients have shown reduced protein synthesis and respiratory chain dysfunction in the presence of this mutation, there were no apparent changes in transcription levels, indicating
that defective transcription termination was unlikely to be the cause of pathogenesis in this disease (Chomyn et al. 1992; King et al. 1992). However, the mutation does cause the production of a new RNA species – RNA19 – which is a partially processed transcript corresponding to the 16S rRNA + tRNA\textsubscript{Leu} + ND1 genes (King et al. 1992). It has been suggested that RNA 19 may be incorporated into mitochondrial ribosomes which causes them to become functionally deficient (Schon et al. 1992). Studies showing that the MELAS mutation reduces the aminoacylation of tRNA\textsubscript{Leu(UUR)} suggest that this might cause reduced association of mitochondrial mRNA transcripts with ribosomes (Chomyn et al. 2000). A comparison of the post-translational methylation of wild-type and mutant tRNAs in both MERRF and MELAS showed that there was no difference in the MERRF phenotype, but a significant decrease in methylation in the MELAS phenotype, suggesting that such post-transcriptional modifications might play a role in MELAS pathogenesis (Helm et al. 1999). Recently, a study showed that MELAS patients apparently lacked a normal taurine-containing modification at the anticodon wobble nucleotide (Kirino et al. 2005).

1.3.2.4 Copy number mutations

Depletion in mtDNA has been shown to be responsible for certain cases of lethal infantile respiratory failure, lactic acidosis and muscle, liver or kidney failure (Wallace 1992). The copy number defects were shown to reduce the amount of mtDNA products, with no corresponding decrease in nuclear-encoded OXPHOS gene products, which in turn led to biochemical deficits. There is some evidence that mtDNA depletion is caused by a nuclear mutation but the exact details are not known (Larsson and Clayton 1995).
### 1.3.3 Mitochondrial dysfunction in neurodegenerative disorders

As the name indicates, neurodegenerative disorders involve the progressive deterioration of neurons in the brain and spinal cord. They affect millions of people worldwide, especially aging populations, and have a wide variety of phenotypic effects including memory loss, dementia and movement disorders like ataxia. Familial protein aggregations are a prominent characteristic of neurodegenerative disorders and specific mutations, that cause defects in certain systemic pathways, have been identified in Alzheimer’s Disease (AD), Parkinson’s Disease (PD), Huntington’s Disease (HD) and Amyotrophic Lateral Sclerosis (ALS) (Swerdlow 2002; Bertram and Tanzi 2005). Most of these disorders also show a dichotomy between the rare familial and the “sporadic” non-familial forms, indicating that the underlying genetic factors and mechanisms of inheritance involved are fairly complex (Bertram and Tanzi 2005).

The greatest risk factor for neurodegenerative diseases such as AD, PD and ALS is aging. The production of ROS by mitochondria, as well as the resulting high mutation rates in mtDNA, are thought to be significant contributors to the aging process and therefore etiologic agents in neurodegeneration (Lin and Beal 2006). In addition, the role of mitochondria in apoptosis has also been implicated in various degenerative disorders.

AD is a late-onset, progressive, age-dependent neurodegenerative disorder that results in the irreversible loss of neurons. It is characterised by the formation of extracellular senile plaques containing the β-amyloid protein. Oxidative damage
and mitochondrial dysfunction have been widely implicated in the pathogenesis of AD (Petrozzi et al. 2007). Oxidative damage occurs early in the AD brain and precedes significant plaque formation. Respiratory chain deficiency has been seen in autopsied brain tissues as well as platelets and fibroblasts of AD patients. The most consistent defect has been reported in COX activity in several regions of the brain (Castellani et al. 2002). There have also been numerous reports of morphological abnormalities in the mitochondria of AD patients (Petrozzi et al. 2007).

PD is a common neurodegenerative disorder characterised by the loss of dopaminergic neurons in the substantia nigra, and the accumulation of intraneuronal inclusions. A possible role for mitochondria in PD was suggested by the discovery that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) acts through the inhibition of Complex I; MPTP is known to induce Parkinsonism in humans, primates and mice (Shults 2004). PD has been associated with large scale rearrangements and with point mutations in mtDNA. (Thyagarajan et al. 2000; Horvath et al. 2007; Petrozzi et al. 2007)

ALS is a motor neuron disease in which there is selective degeneration of the anterior horn cells of the spinal cord and cortical motor neurons. About 20% of familial ALS cases result from mutations in the SOD1 (Cu/Zn-superoxide dismutase) gene (Rosen et al. 1993). It has been suggested that disturbances in mitochondrial function and ROS balance are linked to ALS etiology and studies have shown morphological and metabolic aberrations in the mitochondria of ALS patients (Petrozzi et al. 2007). There are conflicting reports about the exact
changes in respiratory chain activity in ALS. Mutations in the COI gene (Comi et al. 1998) as well as the tRNA\textsuperscript{lle} gene (Borthwick et al. 2006) have been observed in ALS patients. Increased levels of the common 4.9 kb mtDNA deletion as well as multiple mtDNA deletions have also been reported in ALS cases.

1.3.4 Study of mitochondrial dysfunction

1.3.4.1 Laboratory diagnosis

Muscle biopsy is often an early procedure in the diagnosis of mitochondrial disorders. Histochemical studies on skeletal muscle sections include the use of stains such as the modified Gomori’s trichrome, succinate dehydrogenase (SDH), NADH-tetrazolium reductase (NADH-TR) and COX (Filosto et al. 2007). The Gomori trichrome stains ragged-red fibres (RRFs) which are muscle fibres that contain a high percentage of structurally altered mitochondria. RRFs stain red in their sub-sarcolemmal regions where these mitochondria are usually located. SDH and NADH-TR specifically stain RRFs, which are then called ragged-blue fibres. COX-negative fibres are highly suggestive of respiratory chain deficiencies and a combination of SDH (nuclear-encoded) and COX (nuclear and mtDNA-encoded) stains can indicate if the defect is nuclear or mitochondrial in origin. Additionally, sections can be subjected to immunohistochemical analysis using antibodies to mitochondrial-encoded COX subunits.

Histochemical analyses are normally supported by quantitative biochemical studies of the different respiratory chain complexes.
Post-mortem histochemical analysis of CNS tissue is also performed with the stains described above. In general, the histopathological features of CNS tissues in mitochondrial diseases can include neuronal damage, vasculo-necrotic changes, spongy degeneration, gliosis and demyelination (Filosto et al. 2007).

1.3.4.2 Molecular studies of mtDNA mutations

Based on the outcome of clinical investigations of patients suspected of having a mitochondrial disorder, a Polymerase Chain Reaction (PCR)-based sequencing of specific sections of the patient’s mtDNA from blood and muscle biopsies can be employed to identify changes in mtDNA sequence. Any mutations thus identified can be confirmed by using PCRs to check for the presence or absence of specific restriction endonuclease sites. Southern blotting is utilised to determine if the patient’s mtDNA harbours any deletions. These tests are also carried out with frozen or paraffin-embedded brain sections.

Transcription and transcription termination is usually studied using an in vitro system that employs transformed mammalian cell lines like HeLa cells. Translation products can be analysed by using standard approaches such as one- and two-dimensional polyacrylamide gel electrophoresis (PAGE) and Blue Native PAGE, or more sophisticated techniques such as mass spectrometry. Recent approaches have used atomic force microscopy to visualise various aspects of the biogenesis of mitochondria.
1.3.4.3 Cellular studies

Cytoplasmic hybrids (cybrids) are one of the mainstays of mitochondrial research (Khan et al. 2007). They are created by fusing two cells (host and donor) such that their cytoplasmic contents co-exist within a single plasma membrane. A host cell is generally devoid of mtDNA while the donor cell is normally enucleated. Host cells with little or no endogenous mtDNA are created by the continual treatment of these cells with an intercalating agent such as ethidium bromide (EtBr) (King 1996; King and Attardi 1996a). This positively charged dye concentrates in the negatively charged mitochondrial matrix and selectively binds to mtDNA, eventually disrupting its replication. The resulting mtDNA-less cells are termed Rho-0 and survive in culture by the supplementing of culture medium with pyruvate and uridine. Donor cells can be of any type and usually consist of the patient’s platelets or lymphocytes. The fusion is carried out with agents like polyethylene glycol (PEG).

An important aspect of cybrid fusions is that over a period of time, repeated divisions of the cybrid cells dilutes out any non-specific cytoplasmic transfers, meaning that only cells which have received mtDNA will survive in medium that is not supplemented with pyruvate and uridine. Cybrids created through mitochondrial transfer are generally used to study three things:

(i) the phenotypic assessment of the transferred mutation through molecular and biochemical analyses
(ii) the analysis of previously uncharacterised mtDNA, and,
(iii) bigenomic (nuclear and mitochondrial DNA) compatibility.
1.3.4.4 Animal models of mitochondrial disease

There is no simple method as yet to transfer mtDNA alone into cells to generate homoplasmic animal models, although two canine families with a homoplastic mtDNA mutation were recently described (Li et al. 2006). Some of the approaches used to manipulate the mtDNA of mice to generate phenotypes resembling cardiomyopathy and MERRF include (Khan et al. 2007):

(i) introducing exogenous mtDNA into ova and stem cells

(ii) altering nuclear genes to affect the rates of mtDNA mutation

(iii) expressing restriction endonucleases to induce mtDNA deletions, and,

(iv) repeated backcrossing to study mtDNA in different nuclear backgrounds.
1.4 Background to this study

A 33 year old male presented to the Flinders Medical Centre emergency department in 2003 with progressively worsening muscle pain and cramps. The patient noticed orange discoloration to his urine on the day of presentation. Admission creatine kinase (CK) was grossly elevated, with associated alanine transaminase (ALT) and aspartate aminotransferase (AST) elevation but normal alkaline phosphatase (AP) and gamma-glutamyl transferase (GGT). (CK 28, 600 (n<250): ALT 621 (0-50): AST 1600 (n<40): AP 93 (30-110): GGT 27 (0-60)). Myoglobin was not tested in the urine. With supportive therapy all biochemical elevation resolved over one week. The patient was initially investigated for a metabolic myopathy with a forearm lactate test which was normal.

He was the first of three male siblings, born after an uncomplicated term pregnancy. Mild slowing of some motor milestones was noted and in early childhood he received a diagnosis of “cerebral palsy” as his right foot and hand would “turn in” as he walked. Childhood onset strabismus was treated surgically.

The patient recalls cramping and muscle pain and exercise intolerance from childhood that worsened in the months prior to his presentation in 2003. There is no deafness, history of seizures, diabetes or visual complaint.

The second oldest brother has foot cramping but is otherwise healthy, as is the youngest of the three. His mother and maternal grandmother are neurologically normal.
Examination revealed no dysmorphic features or muscle wasting. Hearing, visual acuity and fundoscopy were normal. Ocular movements were normal apart from mild right esotropia. Cerebellar testing was normal. Ankle reflexes required augmentation but otherwise reflexes were normal and the plantar response flexor. Gait demonstrated action dystonia of the right leg and arm. Cardiac examination was normal. Brain MRI demonstrated a left porencephalic cyst and an EEG was unremarkable. A sensory neuropathy was evident on nerve conduction studies.

In 2003, once clinically and biochemically normal, a left vastus lateralis muscle biopsy was performed. Electron microscopy demonstrated prominent subsarcolemmal mitochondrial accumulations but ragged red fibres were not seen. No fibre size variation, inflammation or necrosis was seen. Immunostains for dystrophin I, II and III, sarcoglycans, emerin, dysferlin, beta dystroglycan, spectrin and caveolin were all negative.
Repeat testing of creatine kinase in the last two years has remained normal. A mildly elevated serum lactate (2.1-2.3, n<2) has been noted persistently.

Sequencing of the mitochondrial genome in the patient revealed a novel adenine insertion at nt 3230 in the muscle and blood of the patient (Fig. 1.9). This insertion, hereafter referred to as the 3229.A mutation, is located at the precise junction between the 16S rRNA and the tRNA\textsubscript{LeuUUR} genes.

Earlier studies, especially on the A3243G MELAS substitution, have surmised that mutations in the transcription terminator (mTERF) binding sequence would affect transcriptional regulation. However, this has not been convincingly shown; in one such study, defective protein synthesis was observed but the ratio of the H1 to H2 transcripts remained unchanged (Chomyn \textit{et al.} 1992). The discovery of a second binding site for mTERF at the H1 locus and the demonstration of an mtDNA loop caused by mTERF binding simultaneously to the H1 transcription

![Fig. 1.9 Electropherogram of proband’s muscle mtDNA showing the novel adenine insertion at nt 3230 (red arrow) compared to control mtDNA. The complete mitochondrial genomes from control and proband were sequenced using overlapping sets of primers. This figure was generated from a 646 bp sequence between nt 2850 and nt 3496.](image-url)
initiation and termination sites (Martin et al. 2005) would suggest that insertions at these sites might affect this process and thereby affect transcription termination and re-initiation. This reasoning is strengthened by the fact that human mtDNA is highly compact and butt-jointed and that the tRNA-driven processing of mitochondrial transcripts is extremely precise. The assumption that an insertion at the 16S rRNA-tRNA\textsuperscript{Leu} junction would likely affect transcription termination formed the basis of this study.
1.5 Aims of this study

There were three main aims to this study:

- Analysis of the 3229.A mutation’s pedigree and quantification of its levels 
  *in vivo* and *in vitro*

- Molecular characterisation of the mutation’s effect on the transcription and 
  translation of the human mitochondrial genome

- Study of the mutation’s effect on mitochondrial function (oxidative 
  phosphorylation and programmed cell death)
Chapter II

Materials and Methods
2.1 Materials

2.1.1 Skeletal muscle biopsies

These were obtained with suitable Ethics approval (General Research Application FWA00001785 approved by the Flinders Medical Centre Ethics Committee). Approximately 2-5 mg wet weight each of muscle tissue from consenting control patients undergoing joint replacement surgery at the Repatriation General Hospital, Adelaide, Australia, was obtained and stored at -80°C until further use. Approximately 1 mg of frozen skeletal muscle biopsy tissue from the proband was obtained from the Institute of Medical and Veterinary Sciences (IMVS), Adelaide, Australia and stored similarly. Not all experiments described hereafter could be carried out on these samples due to the paucity of proband muscle tissue.

2.1.2 Cell lines

2.1.2.1 Lymphoblasts

EBV-transformed lymphocytes from the proband and six controls were obtained from the Dept. of Genetic Pathology, Flinders University School of Medicine. These cell lines were maintained in RPMI1640 supplemented with 10% FBS (both from Invitrogen, Mulgrave, Australia), 0.2% sodium bicarbonate (Pfizer, West Ryde, Australia), 0.003% β-mercaptoethanol (Sigma, St. Louis, USA), 100 units/ml penicillin and 100 µg/ml streptomycin (Invitrogen, Mulgrave, Australia), in a 37°C incubator with 5% CO₂.
2.1.2.2 143B206 Rho-zero cells

The mtDNA-lacking 143B206 Rho zero cell line was maintained in DMEM (high glucose) supplemented with 5% FBS, 100 µg/ml sodium pyruvate, 50 µg/ml uridine, 100 units/ml penicillin and 100 µg/ml streptomycin (all from Invitrogen, Mulgrave, Australia).

2.1.2.3 Cybrid cell lines

Trans-mitochondrial cytoplasmic hybrids (cybrids) were prepared using a modification of existing methods (King and Attardi 1996b; Trounce et al. 1996). Approximately $2 \times 10^7$ lymphoblasts from the proband and 6 normal controls were pelleted at 1000x$g$ in a Sigma 6K15 benchtop centrifuge at room temperature. Each pellet was resuspended in 20ml of a 1:1 mixture of RPMI1640 and Percoll (Sigma-Aldrich, St. Louis, USA) that had been equilibrated at 37°C/5% CO$_2$ overnight and Cytochalasin B (Sigma-Aldrich, St. Louis, USA) was added at a final concentration of 20 µg/ml. The cells were then centrifuged at 44000x$g$ for 70 min in a Beckman J2-21 high speed centrifuge using a JA20 rotor with the rotor temperature set to 27°C. The bands corresponding to cytoplasts and a small portion of the band containing karyoplasts were then aseptically removed and transferred to a tube containing approximately 1-2 x $10^6$ previously trypsinised Rho zero cells in 10 ml of the maintenance medium described above. The two sets of cells were co-pelleted at 3000x$g$ for 10 min in the JA20 rotor, following which the medium was aspirated and exactly 1ml of PEG1500 (Roche Applied Sciences, Castle Hill, Australia) was added to the pellet for exactly 1 min. After aspirating off the PEG1500, the pellet was rapidly doused with 10 ml of selection medium (DMEM supplemented with 5% dialysed FBS and antibiotics as above) and 2ml
of the suspension was transferred to a sterile 100mm² Falcon petri dish. 3-4 ml of selection medium was then added to each dish and they were incubated at 37°C/5%CO₂ for 19-21 days. During this time, the selection medium was changed every 2-3 days. Cybrid colonies were picked after 18 days post-fusion and transferred to sterile 25mm² Falcon flasks and grown until confluent. Cybrid cells were maintained in selection medium.

2.1.2.4 Mitochondrial extract preparation

Crude mitochondrial preparations from lymphoblast and cybrid cell lines were obtained as follows:

Approximately 2-3 x 10⁷ cells from the proband and six normal controls were pelleted at 1000 x g in a Sigma 6K15 benchtop centrifuge. The pellets were washed once in 1ml of ice cold STE (250 mM sucrose, 10 mM Tris, 1mM EDTA, pH 7.4, all chemicals from Sigma, St. Louis, USA). The washed pellets were resuspended in 1 ml ice cold STE and transferred to a 45ml nitrogen cavitation cell disruption device (Parr Instruments, Moline, USA) and subjected to compression at 420psi for 10 minutes (Gottlieb and Adachi 2000). The decompressed sample was transferred to a 1.5ml Eppendorf tube and syringed 15-20 times using a 23G needle. Cell debris and the nuclear fraction were removed by centrifuging the samples at 1000 x g for 5 minutes. The supernatants were transferred to fresh tubes and crude mitochondrial pellets were obtained by centrifugation at 10,000 x g for 30 minutes at 4°C. Pellets were resuspended in ice cold STE, aliquotted and stored at -80°C until further use.
Crude mitochondrial fractions were obtained from muscle samples by snap-freezing them in liquid nitrogen followed by powdering in a mortar and pestle and resuspending the disrupted tissue in ice cold STE.

Protein estimation on the mitochondrial extracts was performed by a modified Lowry protocol (Raghupathi and Diwan 1994) and independently reconfirmed using the EZQ protein assay kit (Invitrogen, Mulgrave, Australia).

2.2 Methods

Note: unless otherwise mentioned, all chemicals used in this study were of analytical grade and obtained from Sigma-Aldrich, St. Louis, USA.

2.2.1 Isolation of DNA from blood and cells

Genomic DNA was isolated from venous blood samples, lymphoblasts and cybrids using a standard salting-out procedure (Miller et al. 1988) as follows. Approximately 10ml of heparinised venous blood was freeze-thawed to disrupt RBCs. White blood cells were pelleted at 1000 x g in a tabletop centrifuge and the pellet was resuspended in 9 volumes of Buffer A (0.32M Sucrose, 10mM Tris HCl pH7.6, 5mM MgCl$_2$ and 1% Triton-X100). After incubating on ice for 2-3 min, the suspension was centrifuged at 1000 x g for 15min at 4°C. The resultant pellet was resuspended in 5ml of Buffer B (25mM EDTA pH8.0, 75mM NaCl) to which was added 1% SDS and 0.1mg/ml Proteinase K (final concentration). The suspension was incubated at 37°C overnight, followed by addition of 1.4ml of 6M NaCl. After vortexing the mixture, the tubes was centrifuged at 2500 x g to pellet proteins and the supernatant was transferred to a new tube. Exactly 2 volumes of
room temperature 100% ethanol were added to the supernatant to precipitate out genomic DNA. The DNA precipitate was transferred to a fresh Eppendorf tube and dissolved slowly in 200-300µl of TE buffer (pH 8.0) at 37°C.

Genomic DNA was extracted from cells using the same procedure. In each case, the starting material was approximately 2-4 x 10⁶ cells.

2.2.2 Diagnostic PCR and pedigree analysis by RFLP

Total DNA was extracted from blood samples of the proband, his two siblings, maternal grandmother and father as described above. His mother declined to be tested. In addition, DNA was similarly extracted from the proband’s muscle biopsy, lymphoblasts and cybrid cells. Control DNA was extracted from normal blood samples, lymphoblasts and cybrid cells. Using a standardised protocol, a diagnostic PCR was set up as follows (Yoneda et al. 1996): a 292 bp PCR product between nt 3209 and nt 3500 (as per the Cambridge Reference sequence) was amplified from each sample such that a BfaI site (C'TAG) would be introduced with a mismatch (T-C) at nt 3228 in the forward primer (5’-CCCACCCAAGAACAGGGTCT-3’) with a normal reverse primer (5’-GTGGCGGGTTTAGGGGCTC-3’). A BfaI site at nt 3388 provided the internal control. The PCR reaction volume was 50 µl (25µl PCR reaction mixture + 25 µl 2X Polymate additive from Bioline Pty Ltd (Alexandria, Australia) to increase specificity). The final concentration of MgCl₂ in the reaction was 1.5mM. PCR was carried out at 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 40 sec and 72°C for 30 sec. The last cycle was 94°C for 1 min, 55°C for 45 sec and 72°C for 1 min. PCR products were analysed on a 2% agarose gel stained
with ethidium bromide using standard procedures. Taq polymerase (Biotaq), 10X taq buffer and MgCl₂ were from Bioline Pty Ltd (Alexandria, Australia). All remaining chemicals were from Bio-Rad Laboratories (Pacific) Pty Ltd (Gladesville, Australia).

To address the possibility of accidentally amplifying nuclear-embedded mtDNA pseudogenes using this primer pair, the diagnostic PCR and RFLP was performed on total genomic DNA isolated from mtDNA-lacking 143B206 cells where the absence of any product would serve as a control for the primer pair.

The PCR products thus generated were purified using the SureClean kit from Bioline Pty Ltd (Alexandria, Australia). Approximately 3µg of the purified product was digested with BfaI (New England Biolabs, Ipswich, USA) in the supplied buffer. The enzyme cleaves wild-type products at 2 locations, producing bands of 180 and 112 bp. The presence of the 3229.A mutation generates an additional band of 160 bp, depending on mutant load. The restriction fragments were separated on a 12% polyacrylamide gel using standard protocols and the gels were stained with SYBR Gold (Invitrogen, Mulgrave, Australia) diluted 1:10000 in running buffer (1X TBE). The stained bands were visualized on a GE Healthcare Typhoon scanner. Acrylamide:bis-acrylamide was from Bio-Rad Laboratories (Pacific) Pty Ltd (Gladesville, Australia). Densitometric analysis of the scanned image was performed using ImageQuant 5.2 software (GE, USA)
2.2.3 Measurement of mitochondrial mass

In order to check if there were significant differences in mitochondrial mass between control and proband lymphoblasts and cybrids, 1 x 10^6 cells from each sample were resuspended in 990 µl of their respective media to which 10µl of 2µM MitoTracker Green (Invitrogen, Mulgrave, Australia, final concentration 20nM) was added. Samples were incubated at 37°C for 30 min and analysed on a FACScan-Canto flow cytometer (BD Biosciences, New Jersey, USA). The mean green fluorescence intensity was determined as an average of 100,000 events after correcting for forward and side scatter.

2.2.4 RNA folding

The folding of the mitochondrial tRNA^{Leu(UUR)} gene in the presence and absence of the adenine insertion at the 5’ end was analysed using the mFOLD program (Zuker 2003) (http://mfold.bioinfo.rpi.edu/) with all parameters set at default.

2.2.5 Analysis of mtDNA transcripts

Total RNA was extracted from lymphoblasts, cybrids and muscle samples using a Micro-to-Midi RNA extraction kit (Invitrogen, Mulgrave, Australia) according to manufacturer’s instructions. The quality and quantity of the RNA was measured using a Thermo Scientific Nanodrop 8000 spectrophotometer as well as by electrophoresis on a 1.2% formaldehyde-agarose gel as described (Sambrook and Russell 2001). RNA samples were considered to be pure if the A260/280 ratio was close to 2 and if the 28S:18S ratios visualised by ethidium bromide staining and densitometric analysis was approximately 2:1. First strand cDNA synthesis
was performed using the SuperScript III RT-PCR kit (Invitrogen, Mulgrave, Australia) according to manufacturer’s instructions.

2.2.5.1 Real time PCR

This was performed on a Corbett Life Sciences RotorGene 3000 using the Platinum SYBR Green-UDG Supermix kit (Invitrogen, Mulgrave, Australia). Primers were designed using the PrimerQuest Online Primer Design tool (Integrated DNA Technologies, http://www.idtdna.com/Scitools/Applications/Primerquest/) and checked using Primer-BLAST (NIH, USA, http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi). All qPCR products were designed to be as identical in size as possible. The nuclear 18S and the mitochondrial 12S ribosomal RNA (rRNA) genes were individually used as housekeeping genes. The efficiency of each primer pair was tested using control human cDNA samples serially diluted either two-fold or four-fold. Table 2.1 lists out the various genes analysed along with the primer pairs for each gene (all primers are shown 5’ to 3’). qPCR conditions were as follows: 50° for 2 min (UDG-incubation), 95°C for 2 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 30 sec. Melting curves were measured after a 3 min incubation at 65°C with a range of 65-95°C.
<table>
<thead>
<tr>
<th>GENE</th>
<th>FORWARD</th>
<th>REVERSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S rRNA</td>
<td>CGATAACGAACGAGACTCTG</td>
<td>CATCTAAGGGCATCACAGAC</td>
</tr>
<tr>
<td>12S rRNA</td>
<td>CTAGAGGAGCCTGTTCTGTA</td>
<td>GACCTAAGGTCTTTACGTGG</td>
</tr>
<tr>
<td>H2</td>
<td>GTATTATACCCACACCCACC</td>
<td>GGGTACAATGAGGAGTAGGA</td>
</tr>
<tr>
<td>ND1</td>
<td>TCCTACTCCTCATTGTACCC</td>
<td>TCAGCGAAGGGTTGATAGTA</td>
</tr>
<tr>
<td>ND2</td>
<td>CTTCTGAGTCCAGAGGTTA</td>
<td>GAGAGTGAGGAGAAGGCTTA</td>
</tr>
<tr>
<td>COX1</td>
<td>GCTTCCCTAGGGTTTATCGT</td>
<td>GGGCGAGTCACCTAATAC</td>
</tr>
</tbody>
</table>

Table 2.1 List of primers used to analyse various mtDNA transcripts from the proband and control cDNA samples

2.2.5.2 Northern blotting

Northern blotting (using either upward or downward capillary transfer onto positively-charged nylon membranes (Roche Applied Science, Castle Hill, Australia)) was carried out as described (Sambrook and Russell 2001) using 10 µg per sample of total RNA from the proband and two controls each from lymphoblast and cybrid cells. After denaturing the RNA samples at 60°C for 10 min, they were separated on a 1.2% formaldehyde-agarose gel at 60V for 120 min. The gel was visualised on a UV transilluminator to check for sample integrity and then soaked in 20X SSC (Saline Sodium Citrate, 3M sodium chloride + 0.3 M sodium citrate, pH7) for 20 min at RT. Following overnight transfer onto a nylon membrane, the blot was soaked in 2X SSC for 10 min, air-dried and fixed for 1.5 min on a UV transilluminator. COX1 and 18S rRNA (loading control) DNA probes were generated using the PCR DIG-labeling kit
Using the primers listed in Table 2.1.

The membrane was incubated in pre-hybridisation buffer (DIG Easy-Hyb, Roche Applied Science, Castle Hill, Australia) pre-heated to 50°C, for 30 min at 50°C. Approximately 1µg of each probe was denatured by boiling for 10 min followed by chilling on ice and added to 5 ml of pre-heated Dig Easy-Hyb solution. The pre-hybridisation solution was discarded and the blot incubated with the hybridization buffer for 16h at 50°C. The blot was then subjected to two 5 min low-stringency washes at RT in 50 ml of 2X SSC + 0.1% SDS, followed by two 15 min high-stringency washes at 50°C in pre-heated 0.1X SSC + 0.1% SDS. Following this, the blot was incubated in DIG washing buffer (0.1M Maleic acid, 0.15M NaCl, 0.3%v/v Tween20, pH7.5) at RT for 10 min. It was then blocked at RT for 30 min in 100ml of 0.1M Maleic acid, 0.15M NaCl, pH7.5 containing 1% skim milk powder. After discarding the blocking solution, the blot was incubated with anti-DIG antibody conjugated to alkaline phosphatase (AP) (Roche Applied Science, Castle Hill, Australia) diluted 1:10000 in blocking solution for 30 min at RT. The blot was then subjected to two 15 min washes in washing buffer at RT and equilibrated for 3 min in detection buffer (0.1M Tris.HCl, 0.1M NaCl, pH9.5). Detection of signal was performed using CDPStar (Roche Applied Science, Castle Hill, Australia) diluted 1:100 in detection buffer. Results were visualised on a Fujifilm LAS4000 Luminescent Image Analyser and densitometry was performed with ImageJ software (NIH USA).
2.2.6 Analysis of mitochondrial proteins

2.2.6.1 Immunoblot analysis of COX subunits

Immunoblot analysis of mtDNA-encoded COX subunits I, II and IV was performed using monoclonal antibodies (Molecular Probes, Invitrogen, Mulgrave, Australia, Cat. # A6403, A6404 and A21348 respectively) as follows. Mitochondria were pelleted by centrifuging the crude mitochondrial extracts from control and proband lymphoblast and cybrid cells at 10000 x g for 30 min at 4°C. Mitochondrial proteins were solubilised from the pellets by treatment with 2% SDS (final concentration) at 37°C for 1 hr. The protein concentration in each sample was estimated using a Qubit Fluorometer (Invitrogen, Mulgrave, Australia) according to manufacturer’s instructions. Exactly 50µg of each sample was separated on a 12.5% discontinuous PAGE system (Hoefer Instruments, GE, USA) at 200V for 1h and transferred onto a positively-charged PVDF membrane (Roche Applied Science, Castle Hill, Australia) at 80mA for 2h using a semi-dry electroblotting apparatus (Hoefer Instruments, GE, USA). The blots were probed and processed on a Millipore Snap-ID Western Blotting System according to manufacturer’s instructions, using a Goat α mouse-HRP conjugated secondary antibody (Thermo Scientific, Waltham, USA) and West Pico (Pierce, Rockford, USA) as the substrate for chemiluminescent detection. The results were visualised on a Fujifilm LAS4000 Luminescent Image Analyzer and densitometry performed with ImageJ software (NIH USA). Acrylamide:bis-acrylamide was from Bio-Rad Laboratories (Pacific) Pty Ltd (Gladesville, Australia).
2.2.6.2 Blue-Native PAGE analysis of mitochondrial proteins

Mitochondrial respiratory chain holocomplexes were solubilised from crude mitochondrial preparations by adding dodecyl maltoside at a final concentration of 1.5%. Exactly 20µg of each sample was separated on a 4-16% native polyacrylamide gel as described (Schagger and Von Jagow 1991) using the NativePAGE Novex Bis-Tris Gel system (Invitrogen, Mulgrave, Australia) according to manufacturer’s instructions. After fixing and destaining the gel, it was scanned on a GE Healthcare Typhoon 9400 and densitometry was performed with ImageJ software (NIH USA). The identity of each holocomplex was confirmed using mass spectrometry as follows.

Individual holocomplex bands were excised from the gel, washed and incubated in 100 µl of 100 mM Ammonium Bicarbonate with shaking at ambient temperature for 30 min. The wash process was repeated three times and the plugs allowed to dehydrate overnight. They were then rehydrated in 20 µl of 50 mM Ammonium Bicarbonate containing 400 ng Trypsin Gold (Promega, Alexandria, Australia) and the proteins digested for 4h at 37°C. The digested peptides were sequenced with a Thermo LTQ XL linear ion trap mass spectrometer fitted with a nanospray source (Thermo Electron Corp., Waltham, USA). The samples were applied to a 300 µm i.d. x 5 mm C18 PepMap 100 precolumn and separated on a 75 µm x 150 mm C18 PepMap 100 column using a Dionex Ultimate 3000 HPLC (Dionex Corp., Bannockburn, USA) with a 55 minute gradient from 2% acetonitrile to 45% acetonitrile containing 0.1% formic acid at a flow rate of 200 nl/min followed by a step to 77% acetonitrile for 9 minutes. The mass spectrometer was operated in positive ion mode with one full scan of mass/charge
(m/z) 300-2000 followed by product ion scans of the 3 most intense ions with dynamic exclusion of 30 seconds and collision induced dissociation energy of 35%. The MS spectra were searched with Bioworks 3.3 (Thermo Electron Corp., Waltham, USA) using the Sequest algorithm against the IPI Human database v3.39 using Trypsin digestion as the protease and allowing for two missed cleavages, with the following filters: 1) the cross-correlation scores (Xcorr) of matches were greater than 1.5, 2.0 and 2.5 for charge state 1, 2 and 3 peptide ions respectively, 2) peptide probability was greater than 0.001 and 3) each protein identified had at least 2 different peptides sequenced. The mass tolerance for peptide identification of precursor ions was 1 Da and 0.5 Da for product ions.

2.2.7 Analysis of respiratory chain activity

Standardised spectrophotometric assays of citrate synthase and respiratory chain complexes I, II + III and IV (Trounce et al. 1996) were performed with a few modifications on a UV-3000 spectrophotometer (Shimadzu Instruments, Japan). Enzyme activities were generally calculated as nmol/min/mg protein, except for complex IV (cytochrome c oxidase) whose activity was expressed as a first-order rate constant ($k$/min/mg protein). All respiratory chain activities are expressed relative to citrate synthase activity to correct for differences in mitochondrial mass.

2.2.7.1 Complex I (NADH ubiquinone oxidoreductase)

In this assay, the oxidation of 50 µM NADH was monitored at 340 nm in a 1 ml cuvette containing 50 µg mitochondrial protein in a final volume of 1 ml reaction mixture containing 31.25 mM potassium orthophosphate buffer, 12.5 mM MgCl$_2$, 50 µM NADH.
1.25 mM KCN and 3.125 mg/ml fat-free BSA. The reaction was carried out for 3 min at 30°C and inhibited by the addition of 5 µg rotenone.

2.2.7.2 Complex II + III (Succinate-Cytochrome C oxidoreductase)
Here the reduction of cytochrome c is monitored at 550 nm, 30°C, in a 1 ml cuvette. Crude mitochondrial extracts containing 50 µg protein in hypotonic buffer (25 mM potassium phosphate) were subjected to five freeze-thaw cycles using liquid nitrogen. Sodium succinate (20mM final concentration) and 2 µg/ml rotenone were added to the disrupted extracts which were incubated for 10 min at 30°C. The reaction was initiated by the addition of cytochrome c and followed for 3-5 min.

2.2.7.3 Complex IV (Cytochrome c oxidase)
This assay involved measuring the oxidation of reduced cytochrome c at 550nm, 30°C, in a 1 ml cuvette. A 0.02 g/ml stock of cytochrome c in 10 mM potassium phosphate buffer was prepared and 0.05 g/ml of sodium dithionite was added to it to reduce the cytochrome c. The ratio of absorbance at 550 and 565 nm was measured over an 18h period, following which the dithionite was removed by dialysis in 500 ml of 10 mM potassium phosphate buffer. Dialysis was carried out for 24 h, with at least four changes of the buffer during that period. The reaction was initiated by adding 20 µm reduced cytochrome c to 10 mM potassium phosphate buffer, following which 50 µg mitochondrial protein previously solubilised in 2% Triton- X 100 was added to the reaction mixture. The reaction was followed for 5 min.
2.2.7.4 Citrate synthase

The activity of this mitochondrial matrix marker enzyme was monitored by following the reduction of 5,5’-dithiobis(2-nitrobenzoic acid) (DTNB) at 412 nm at 30°C in a 1 ml cuvette. 50 µg of mitochondrial protein was solubilised with 10% Triton-X 100 following which the reaction mixture of 0.1 mM DTNB in 0.1M Tris-HCl (pH 8.0) and 0.3 mM Acetyl-CoA was added. The final volume of 1ml was made up with deionised water and the reaction was initiated by the addition of 0.5 mM oxaloacetate and followed for 1-3 min.

2.2.7.5 Complex V (ATP synthase)

This assay is based on the production of ATP from a Luciferase-luciferyl-ADP complex which results in a release of luciferase, an activity that is monitored at 560 nm. The assay was carried out in a black 96-well ELISA plate and luminescence was measured using a Beckman-Coulter DTX-880 Multimode plate reader fitted with the appropriate filters. ATP synthesis was measured in proband lymphoblast and cybrid cells with at least two controls for each. The cells were solubilised with 50 µg/ml digitonin for 1 min at RT with gentle shaking.

The assay was carried out in quintuplicate, with three replicates used to measure ATP synthesis and two used to measure baseline luminescence corresponding to non-mitochondrial ATP production. Each replicate well on the plate utilised 1.5 x 10^6 digitonised cells resuspended in 160 µl Buffer A (150 mM KCl, 25 mM Tris-HCl, 2 mM EDTA, 0.1% BSA, 10 mM potassium phosphate, 0.1 mM MgCl_2, pH 7.4). To this was added 5 µl P_1, P_5-di(adenosine) pentaphosphate (0.15 mM0, 5 µl sodium succinate + 1 µl of 2 µg/ml rotenone, 10 µl Buffer B (0.5M Tris-acetate
pH 7.75, 0.8 mM luciferin, 20 µg/ml luciferase, premixed and incubated on ice for 10 min prior to addition) and 5 µl ADP (final concentration 0.1 mM). For the two baseline controls, 2 µl of oligomycin (final concentration 2 µg/ml) was added. The volumes in each well were made up to 200 µl with Buffer A. After a mild vortexing of the plate, readings were measured at 10 sec intervals for 7-8 min. Protein concentrations of digitonised samples was measure using the Qubit fluorometer (Invitrogen, Mulgrave, Australia) according to manufacturer’s instructions.

2.2.8 Analysis of apoptosis and oxidative stress

This was performed on proband lymphoblast and cybrid cells with at least two controls for each, using readily available commercial kits.

2.2.8.1 Apoptotic DNA ladder assay

This assay was carried out using the Roche Apoptotic DNA ladder kit (Cat. No. 11 835 246 001). Approximately 2 x 10⁶ cells from each sample were resuspended in sterile 1X PBS (pH 7.4). Total DNA was then extracted using components from the kit according to manufacturer’s instructions. DNA concentrations were measured using the Qubit fluorometer (Invitrogen, Mulgrave, Australia) according to manufacturer’s instructions. Approximately 3 µg of each DNA sample was loaded onto a 1% agarose gel. The positive control from the kit was loaded alongside, along with a molecular weight marker. Electrophoresis was carried out at 70V for 1.5 h in 1X TBE buffer. DNA was visualised by staining with ethidium bromide as described earlier.
2.2.8.2 Measurement of 8-OHdG levels

This was performed using the SKT-120 ELISA kit (StressMarq Biosciences Inc., Victoria BC, Canada). Exactly 3 µg of each DNA sample from above was diluted to a final volume of 100 µl in 0.59% Barbital Acetate buffer (pH 5.3) and treated with Nuclease P1 (Sigma, St. Louis, USA) for 2 h at 70°C. The pH was then adjusted to 7.0 by adding 20µl of 1M Tris.Cl (pH 8.0) to each sample, followed by the addition of ~ 3 units each of Antarctic phosphatase (NEB, Ipswich, USA) in the supplied buffer. Samples were incubated at 37°C for 30 min, followed by boiling for 10 min and placed on ice till further use. The digested samples were diluted either 1:10 or 1:100 in the buffer supplied with the kit and the ELISA was performed according to manufacturer’s instructions. Results were measured on a Beckman-Coulter DTX-880 Multimode plate reader fitted with the appropriate filter.
Chapter III

Preliminary Analysis of the 3229.A Mutation
3.1 Introduction

The first steps in analysing a novel mtDNA mutation often involve studying its tissue distribution, load and pedigree. Most pathological mutations tend to occur in a heteroplasmic state and the mutant and wild-type (wt) mtDNA’s coexist in differing proportions in different tissues in the proband, leading to the clinical features of mitochondrial disease (Yoneda et al. 1996). There are two notable exceptions to this scenario (Horvath et al. 2009; Yu-Wai-Man et al. 2009). Leber’s Hereditary Optic Neuropathy (LHON) has been associated with three homoplasmic point mutations – the G3460A, G11778A and T14484C substitutions. Disease penetrance has been thought to arise not only from the mutations but also from nuclear gene effects and environmental factors (Yu-Wai-Man et al. 2009). In infantile reversible COX deficiency, studies have shown strong evidence that the homoplasmic T14674C point mutation has a pathogenic role in the disease (Horvath et al. 2009).

Blood samples or hair follicles are commonly used in the preliminary molecular analysis of mitochondrial DNA. Through mtDNA sequencing, the proband’s blood showed the presence of a novel 3229.A mutation. Since he presented with symptoms consistent with a mitochondrial myopathy, a skeletal muscle biopsy was performed and sequencing the mtDNA in his muscle tissue also showed the mutation. The maternal pattern of inheritance is studied by analysing tissues from the proband’s family members (when possible) and constructing a simple pedigree chart. In this case, blood samples were obtained from consenting family members to detect the presence or absence of the 3229.A mutation.
Early studies used denaturing urea polyacrylamide gels to detect single nucleotide polymorphisms in mtDNA (Ruiz-Pesini et al. 2007). With the advent of modern and highly sensitive methods such as PCR (polymerase chain reaction), a commonly used experimental procedure to confirm the presence of an mtDNA point mutation as well as analyse its load is the PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism). This involves amplifying a specific portion of the proband’s mtDNA, digesting it with a restriction enzyme and separating the resulting fragments on an agarose gel. There are two ways in which the PCR can be designed (Yoneda et al. 1996):

(i) if the point mutation introduces or abolishes a restriction site, then the mutant PCR product, when digested with that enzyme will either be cleaved or remain uncut. This is seen, for example, in the A3302G (Maniura-Weber et al. 2006) and the T9035C (unpublished observations) substitutions, both of which introduce a \textit{DdeI} restriction site (C^TNAG) and in the A8344G MERRF mutation which produces a CViJI site (A^GCC) (Shoffner et al. 1990). The PCR product is designed to include an existing (control) site for the same enzyme.

(ii) when no restriction site is created by the mutation, PCR-mediated RSM (restriction site modification) is used. Here a mismatch primer is designed to introduce a restriction site that has an internal control downstream of the mutation.
The PCR-RSM approach was used to analyse the 3229.A mutation. As Fig. 3.1 illustrates, the forward primer contained a T>C mismatch at nt 3228, creating a BfaI site (C^TAG) in the presence of the mutation. Wild-type (Wt) samples would therefore only be digested by the enzyme at the internal control site and produce fewer restriction fragments.

Since the 3229.A mutation is located at the exact breakpoint between the 16S rRNA and tRNA\textsubscript{Leu(UUR)} genes, I surmised that its presence might have some effect on the secondary structure of the mature RNA transcript(s). Since the wt polycistron is cleaved at nt 3229, it was assumed that the insertion would now form the 5’ end of the tRNA\textsubscript{Leu(UUR)} transcript and its folding was analysed by the online tool mFOLD (Zuker 2003), which determines the free energy associated with different RNA conformations. With a novel insertion such as this one, it is not possible to perform bioinformatic analyses that look at evolutionary conservation of the mutation.

Once the presence of the mutation has been confirmed, it is important to analyse whether it affects mitochondrial mass and number. The approach chosen here was
to use the mitochondrial-selective fluorescent dye MitoTracker Green (MTG) (Poot et al. 1996) that is lipophilic and accumulates within mitochondria, and contains a mildly thiol-reactive chloromethyl moiety that keeps it associated with the organelle even if the cells are subsequently fixed. The use of flow cytometry enables one to estimate both mitochondrial mass (strength of signal) as well as number (average of total events).

3.2 Results

3.2.1 Analysis of the pedigree, load and tissue distribution of the 3229.A mutation

The PCR-RSM assay was performed with the following samples:

(i) proband’s blood and muscle

(ii) blood samples from proband’s family members, including his two brothers, maternal grandmother and father.

(iii) cell lines derived from the proband’s lymphocytes as well as trans-mitochondrial cybrids harbouring the mutation

(iv) the mtDNA-lacking 143B206 osteosarcoma cell line

The BfaI restriction digestion pattern of the 292 bp PCR products, along with the corresponding pedigree chart is seen in Fig. 3.2. If the mutation is heteroplasmic, we expect to see bands of 180 bp, 160 bp and 112 bp. The 160 bp fragment arises from the cleavage of the 180 bp fragment at the mutation site; the 20 bp run-off fragment is not shown. The 180 bp band will be absent if the mutation is homoplasmic while the 160 bp band will not show up in a wt sample. The 112 bp
band is formed by the internal control restriction site, which serves as an indicator of the efficiency of digestion. Partial digestion of a PCR fragment with a single cut site and no control site could lead to erroneous results; partial digestion with an internal control site would not affect the Wt:mutant ratio measurement and would enable visualisation of any uncut material resulting in the digestion being repeated. The percentage load is normally calculated by measuring the density of each band and dividing that by the combined densities of all the bands in each lane. However, heteroduplexes of mutant and wild-type sequences that may form during the last annealing step of the PCR reaction may not be completely digested by the restriction enzyme, leading to erroneous calculation of the percentage of heteroplasmy (Shoffner et al. 1990; Yoneda et al. 1996). One way to overcome this is to use a radioactive label in the last annealing step – the only products that will be labeled are homoduplexes which would accurately reflect the Wt:mutant ratio (King et al. 1992). Since my laboratory was not equipped to use radioactivity, I chose to correct for uncuttable heteroduplexes by using the square root of the observed heteroplasmy expressed as a decimal fraction (Shoffner et al. 1990; Yoneda et al. 1992).
Fig. 3.2  Analysis of mutant load and pedigree by PCR-RSM. 292 bp PCR products from the tissues of proband and family members were digested with BfaI and separated on a 12% polyacrylamide gel. The arrow indicates the diagnostic 160 bp band produced by the 3229.A mutation. Dotted lines in the pedigree chart indicate untested samples. The pedigree shows a clear pattern of mitochondrial (maternal) inheritance. The proband is homoplasmic for the mutation in his muscle (M) and has an ~ 78% mutant load in his blood and lymphoblasts (B, L). His siblings (B1, B2) and maternal grandmother (MG) are homoplasmic or near-homoplasmic. His father (F) does not carry the mutation.
Fig. 3.3 Analysis of the mutation load in cell lines. The mtDNA-lacking osteosarcoma Rho-zero (143B206) cell line did not yield any PCR product as expected. The trans-mitochondrial cybrid generated from the proband’s lymphoblasts (CYB216) were homoplasmic for the 3229.A mutation (the arrow indicates the diagnostic 160 bp band).
3.2.1.1 Proband

The proband’s muscle and blood (Fig. 3.2, Lanes M and B respectively) definitely contain the mutation. His blood is heteroplasmic for the mutation with a load of ~78%. He is homoplasmic for the mutation in his muscle.

3.2.1.2 Proband’s family members

The mutation is present in both the proband’s siblings as well as his maternal grandmother (Fig. 3.2, Lanes B1, B2 and MG respectively). While B2 and MG are homoplasmic for the mutation, its load in B1 is >95%. It is absent in his father. His mother declined to be tested.

3.2.1.3 Cell lines

In lymphoblasts derived from the proband (Fig. 3.2, Lane L), the mutation load is ~78%. The 143B206 cell line does not show any PCR products, as expected for a cell line that does not possess mtDNA while the trans-mitochondrial cybrid derived from fusing the proband’s enucleated lymphoblasts with 143B206 cells is seen to be homoplasmic for the mutation (Fig. 3.3).

The quantification of the mutation load in various samples has been summarised in Table 3.1.
<table>
<thead>
<tr>
<th>PROBAND SAMPLE</th>
<th>MUTATION LOAD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle</td>
<td>100</td>
</tr>
<tr>
<td>Blood</td>
<td>~78</td>
</tr>
<tr>
<td>Lymphoblast</td>
<td>~78</td>
</tr>
<tr>
<td>Cybrid</td>
<td>100</td>
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</tbody>
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<table>
<thead>
<tr>
<th>FAMILY MEMBER</th>
<th>MUTATION LOAD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Siblings</td>
<td>~100</td>
</tr>
<tr>
<td>Maternal grandmother</td>
<td>100</td>
</tr>
<tr>
<td>Father</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 3.1  Summary of the tissue distribution and percentage of load of the 3229.A mutation in the proband and family members.
3.2.2 Estimation of mitochondrial mass and number

Using equal numbers of cells from each control and from the proband, MTG staining followed by flow cytometry showed no significant differences in mitochondrial mass or number between the control and proband samples in either lymphoblasts or trans-mitochondrial cybrids (Fig. 3.4). The mean number of events analysed was 100,000 and any significant change in mitochondrial mass or the number of mitochondria per cell would have been reflected in the mean green fluorescence. The number of controls used was six in the case of each cell type.

3.2.3 Prediction of tRNA<sub>Leu(UUR)</sub> secondary structure

The result of the mFOLD analysis of wt and mutant tRNA<sub>Leu(UUR)</sub> gene sequences and the predicted structures along with the energy changes (dG) are shown in Fig. 3.5. The DNA sequences, with and without the extra 5’ adenine, were input into the mFOLD server which predicted that in each case, there were two possible conformations for the putative RNA molecule – a circular structure and a more conventional “tRNA”-like structure. Of the two, the circular structures in both wt and mutant mtDNA have an identical dG of -4.44, indicating that the adenine insertion has no effect on the stability of the molecule. The cloverleaf structure shows a 12.5% shift toward a less energy-efficient state when the mutation is present.
Fig 3.4 Measurement of mitochondrial mass by MTG staining. No significant differences were observed between controls (n=6 in each case) and proband lymphoblasts or cybrids. The error bars indicate +/- 2SD.
Fig. 3.5 mFOLD analysis of tRNA$^{\text{Leu(UUR)}}$ secondary structure formation and energy change. The predicted cloverleaf structure shows an ~13% shift towards a less favourable energy state in the presence of the 3229.A mutation (Mut) as compared to wild-type (Wt). The DNA sequences for the tRNA$^{\text{Leu(UUR)}}$ gene were input into the mFOLD server with all parameters set at default.
3.3 Discussion

The 3229.A mutation bears the typical hallmarks of a mitochondrial (maternal) pattern of inheritance of point mutations. It is present in the proband, his siblings and his maternal grandmother but absent in his father. One could argue that the mother was not tested but it is known that a mother who is homoplasmic for an mtDNA mutation will transmit only mutated mtDNA to her offspring (Larsson and Clayton 1995). Since the maternal grandmother is homoplasmic for this mutation in her blood, it follows that she passed it onto her daughter who in turn passed it onto her children. Given that all three siblings show exceedingly high levels of the mutation, one can assume that there were no bottlenecks during oogenesis or embryogenesis (Larsson and Clayton 1995) that caused a significant variation in the transmission of this mutation and that the proband’s mother is likely to be homoplasmic for it. There are studies that show that a mother who is heteroplasmic for an mtDNA mutation does not always transmit it to her children, as seen in a case of Kearns-Sayre syndrome (KSS) (Larsson et al. 1992). However, with some rare exceptions (Ozawa et al. 1988; Shanske et al. 2002), large-scale deletions such as those seen in KSS are sporadic and never transmitted (Zeviani et al. 1990), while point mutations invariably are (Wallace 1992). The presence of heteroplasmic mtDNA in the proband and one of his siblings does imply that the mother may be heteroplasmic but without actually testing her mtDNA, it is only possible to speculate about the origin of the heteroplasmy. Given that a number of mtDNA mutations show variability in both tissue distribution and mutation load (Ruiz-Pesini et al. 2007), one possibility is that the grandmother and/or mother had heteroplasmic mtDNA in their ova. An
unfortunate limitation of such pedigree studies is that often only one tissue sample (normally blood as in the case of the grandmother) or none (as in the case of the mother) is available to study so the mutation load in different tissues has to be inferred.

A number of factors are involved in determining the pathogenicity of an mtDNA mutation including tissue distribution, the oxygen requirements of the tissue, percentage of heteroplasmy, mtDNA copy number and how it affects mitochondrial function. For example, point mutations in mitochondrial genes that encode the various respiratory chain subunits can cause a deficit in OXPHOS, leading to a deleterious effect on tissues like muscle or organs like the heart, spleen and kidneys that require large amounts of energy (Wallace 1999). mtDNA has a very high rate of mutation; cells are initially heteroplasmic but tend toward homoplasm. This drift induces a pathogenic effect on cellular bioenergetics, especially as the mutation load increases (Wallace 1999). However, different mutation loads can have differing pathogenic effects even within the same mitochondrial disease. For example, the A3243G MELAS mutation can cause myopathy and stroke-like activity when present in >85% of mtDNAs but induces maternally inherited diabetes and deafness when present at levels ranging from 5-30% (Goto et al. 1990; Wallace 1999). Other mitochondrial diseases such as LHON and CNS diseases such as AD/PD are associated with consistently homoplasmic mtDNA mutations (Wallace et al. 1988; Shoffner et al. 1993). In the case of our proband, it appears as if homoplasm is more likely to have a pathogenic effect since his muscle is homoplasmic for the 3229.A mutation and he presents with a case of myopathy. Given his late onset of the disease, it may be
argued that this mutation is not very pathogenic but without neurological analysis of his siblings, mother and maternal grandmother, it is again only possible to speculate about its pathogenicity. As mentioned earlier, the molecular basis of mitochondrial disease is complex and poorly understood. The onset of many mtDNA diseases is delayed and aging has been implicated in the progression of disease severity (Wallace 1999). It is possible that aging may exacerbate the proband’s myopathy and cause other organ systems to exhibit a mutation load-dependent pathology. However, this reasoning is purely inductive since, as mentioned above, there is often poor correlation between mutation load or distribution and disease severity.

As has been extensively discussed in the introductory chapter, mitochondrial defects have been implicated in a wide variety of neurodegenerative disorders, aging and cancer. Depending on their location, point mutations have been known to affect mitochondrial biogenesis, respiration, free radical homeostasis and apoptosis. One of the earliest reports of a mitochondrial role in hypermetabolism was in a patient with increased numbers of abnormal mitochondria in her skeletal muscle (Luft et al. 1962) which led to the term “mitochondrial myopathy” being coined (Wallace 1999). Here we clearly have a mutation present in every copy of the mtDNA genome in the proband’s muscle but there is no significant increase in mitochondrial mass or number. This would imply that any mitochondrial-related pathophysiology in this myopathy arises from other causes.

Given the location of the mutation, it is likely that if affects mitochondrial biogenesis, either at the transcriptional or translational level, or both. The “tRNA
punctuation” model of processing the mtRNA polycistron (Ojala et al. 1981) dictates that a precise endonucleolytic cleavage occurs on either side of the butt-jointed mtDNA genes once the tRNAs have folded into their cloverleaf structures. This would suggest that even a single base insertion can disrupt this process and the predicted shift to a less-favourable energy state in the tRNA_{Leu(UUR)} molecule in the presence of the mutation would suggest that there is a high likelihood of this occurring. Even assuming that the cleavage occurs normally, the unstable tRNA molecule would likely be unable to participate in translation, leading to a deficit in mitochondrially-encoded protein subunits. Secondly, the extra base could change the dynamics of mTERF binding to the transcription termination site leading to an abnormal increase in mtDNA transcripts, or affect the conformation of the mtDNA loop formed by mTERF binding to both transcription initiation and termination sites, leading to a significant reduction in mtDNA transcription.

In conclusion, preliminary analysis of the novel 3229.A mutation suggests that a detailed molecular analysis of mtDNA transcription and translation in the proband is required, which is the focus of the next chapter.
Chapter IV

Molecular Analysis of the Effect(s) of the 3229.A Mutation on mtDNA Transcription and Translation
4.1 Introduction

This chapter details the investigation into whether the 3229.A mutation interferes with mtDNA transcription and if so, what effect that would have on translation of the mitochondrial transcripts.

4.1.1 Analysis of mtDNA transcription

Early studies on the transcription termination region of mtDNA focused on identifying and isolating the transcription termination factor mTERF. Once it was established that mTERF was responsible for the 15-60 fold difference between levels of mt-rRNAs and mt-mRNAs, work began to focus on mutations within the mTERF binding site. One of the best-known such mutations, the A3243G MELAS substitution, is located within the tridecamer sequence that mTERF binds to. Two studies that looked at whether the mutation would impair transcription termination came to different conclusions (Hess et al. 1991; Chomyn et al. 1992). Chomyn et al observed no major difference in the steady state levels of mt-rRNAs and an almost unchanged ratio of rRNA to mRNA. Hess et al concluded that the MELAS mutation would severely impair transcription termination at the 16S rRNA-tRNA_{Leu} boundary, which in turn would affect tRNA_{Leu} structure and function. However, neither of these studies looked at actual individual mtDNA transcripts but focused on protein-DNA interactions or transcription run-off assays to quantify transcription termination. Hess et al also discovered additional mTERF binding sites in the D-loop and postulated that these could be alternate points to override impaired mTERF binding to its recognition sequence in the tRNA_{Leu} gene. It was not until 2005 that it was established that the D-loop binding
sites of mTERF were in fact transcription initiation sites and that mTERF binds to both sites, forming an mtDNA loop that might be involved in the recycling of mitochondrial transcription machinery (Martin et al. 2005).

Since it was apparent that mutations in the vicinity of the mTERF termination binding site would tend to disrupt mtDNA transcription termination, I chose to follow the hypothesis that the 3229.A insertion would affect mTERF-DNA binding and impair transcription termination. As mentioned above, early studies of mtDNA transcription utilised run-off assays or S1-protection assays to visualise truncated transcripts (Montoya et al. 1983; Hess et al. 1991). With the advent of more modern and highly sensitive methods like real-time PCR, it is possible to accurately quantify specific transcripts in a sample even if they are expressed at very low levels. Since the older methods might not have been sensitive enough to detect very subtle changes in transcription termination, I decided to analyse individual mtDNA, transcripts on either side of the insertion, by real-time RT-PCR (reverse transcriptase-polymerase chain reaction) and Northern blotting.

In the measurement of RNA levels, there are two important parameters to account for in both traditional blotting methods and newer techniques like real-time PCR – the quality of the template prepared and the use of an accurate standard. To address the first issue, I decided to use readily available components that yielded high-quality samples in a reproducible manner. In the spin-column based Invitrogen PureLink total RNA extraction kit, the proprietary lysis buffer contains guanidium isothiocyanate, a chaotropic salt that protects the sample RNA from endogenous RNAses. Using this kit, I was able to successfully and reproducibly
extract ~70-80 µg of high-quality total RNA from 5 x 10⁶ cells. I ensured that the preparation was DNA-free by performing an on-column DNAse I treatment according to the PureLink kit instructions.

There are two possible ways of quantifying RNA using real-time PCR – absolute or relative (Pfaffl 2001). The first method involves referencing an internal or external calibration curve, while relative quantification is based on comparing the expression of a target gene to a reference gene. Since I was interested in estimating the relative levels of mt-rRNA in relation to the downstream mRNAs, I chose to adopt the second approach. However, the choice of a reference gene is often the most difficult aspect of real-time PCR, especially since many supposedly stable house-keeping genes are themselves up- or down-regulated *in vitro* (Pfaffl *et al.* 2004). I decided to use two different reference genes – the endogenous MT-RNR1 (12S rRNA) (RefSeq NC_012920) and the nuclear-encoded 18S rRNA (RefSeq NT_167214.1). The latter is a popular housekeeping gene in RNA analysis and would also serve as a loading control in the Northern blot experiments. The use of an endogenous, co-amplified reference gene like the mitochondrial 12S rRNA is advantageous in that target and reference are equally affected by the extraction procedure and reverse transcription. An earlier study has shown that the use of 12S rRNA as a reference gene to estimate COX I RNA levels is reliable and reproducible (Harting and Wiesner 1997).

While real-time PCR is a sensitive method for the detection and quantification of gene expression, especially with low-abundance RNAs, most of the published models for calculating expression levels can only utilise one reference and one
target gene. Since I intended to use two house-keeping genes and study the expression of multiple transcripts in at least two controls plus the proband, I decided to adopt the powerful Relative Expression Software Tool (REST) (Pfaffl et al. 2002) which allows a group-wise comparison of the data. In it, the relative expression of a target gene is computed based on the efficiency of the reaction and the crossing point (CP or Ct) difference of an unknown sample versus a control or controls. The equation it uses is shown below (Fig.4.1). REST not only eliminates any errors in calculation due to differing efficiencies in each PCR tube but also has a version specifically designed to import the data from the thermal cycler that I was using (REST-RG for the RotorGene 3000).

I chose to use SYBR Green I in the real-time PCR (qPCR) assay. It is a relatively inexpensive, sensitive fluorescent dye that binds to double-stranded DNA

\[
R = \frac{(E_{\text{target}})^{\Delta CP_{\text{target}} \ (\text{MEAN control} - \text{MEAN sample})}}{(E_{\text{ref}})^{\Delta CP_{\text{ref}} \ (\text{MEAN control} - \text{MEAN sample})}}
\]

Fig.4.1 The Pfaffl equation used in REST to calculate the relative expression (R) of a target gene (E = efficiency, CP = crossing point)
(dsDNA) in a directly proportional manner and its signal is detectable on most real-time PCR instruments (Ishiguro et al. 1995; Wittwer et al. 1997). It is compatible with melting curve analysis. The qPCR was designed to measure the levels of the following mitochondrial transcripts – H2 (almost genome length polycistron), ND1, ND2 and COX I. The design, including nucleotide positions of each product, is summarised in Fig. 4.2 below. As can be seen, the primers for the H2 transcript bind on either side of the transcription termination break point, ensuring that only a non-truncated transcript is being measured. The lack of introns in human mtDNA ensures that the product can be amplified without any problems. Each qPCR primer pair (including that of the 137 bp 18S rRNA product) was designed to amplify a product as close in size as possible to the others, while still possessing a predicted primer efficiency >99%. Every product from each reaction was always checked on an agarose gel for purity.

Fig.4.2  The mitochondrial genes analysed by qPCR. H2 refers to the almost genome-length polycistron and the dotted line represents the 16S rRNA-tRNA^Leu^ boundary (the transcription breakpoint after termination).
4.1.2 Analysis of mt-mRNA translation

The respiratory chain complexes each consist of several sub-units, some of which are encoded by mtDNA. It follows that any changes to the mtDNA transcript would affect the levels of those sub-units and likely affect the assembly of the mitochondrial holocomplexes. I decided to utilise Blue-Native PAGE (polyacrylamide gel electrophoresis, BN-PAGE), an elegant and powerful protocol to visualise and measure holocomplex levels (Schagger and Von Jagow 1991). Protein complexes are solubilised with mild neutral detergents following which they are treated with Coomassie blue G250. They are separated based on the net negative charge that the dye confers to the protein surface and migrate to the anode based on molecular mass and/or size. This assay is useful to separate protein complexes from 10-10000 kDa. I decided to identify the holocomplexes by mass spectrometry and perform a densitometric analysis of the separated proteins.

COX deficiency has been implicated in mitochondrial myopathy (Wallace 1999). To investigate whether there were changes in the levels of individual mtDNA-encoded protein sub-units, I measured the levels of two sub-units of COX (CO I and CO II) by immunoblotting, using the nuclear-encoded CO IV as an endogenous control.
4.2 Results

The different assays were performed with RNA or protein from lymphoblasts and cybrid cells. Due to a paucity of muscle tissue, I could not perform any assays other than qPCR with it.

4.2.1 Analysis of mtDNA expression by real-time RT-PCR (qPCR)

To analyse the efficiency of each primer pair used in the qPCR reactions, standard curves were prepared with serial 2-fold dilutions of control cDNA samples. Each primer pair was found to be close to the ideal value of 1 in terms of efficiency (Fig. 4.3). The error values for each reaction were less than 2%. The purity and quality of the different qPCR products was investigated at the end of a run by melting curve analysis and visually (through agarose gel electrophoresis). As shown in Fig. 4.4, ethidium bromide staining of the separated products does not show any impurities or artifacts such as primer dimers and/or partially amplified species in any of the samples. The product sizes are of the expected values and none of them show any degradation. Melting curve analysis was performed between 65 and 90°C (Fig. 4.5) and further confirms the purity of the reaction. There are distinct single peaks above the threshold for each product close to the predicted melting temperature (Tm). The lack of multiple peaks, especially at the lower end of the temperature scale, indicates that there was no non-specific amplification in any of the reactions.
Fig. 4.3 Standard curve for each qPCR primer pair. Each set of primers shows close to 100% efficiency in amplifying serial 2-fold dilutions of control cDNA samples.
Fig. 4.4 Quality and purity of qPCR products. Samples were separated on a 2% agarose gel to check for contaminants and to ensure product sizes were accurate. Products from the following samples are shown: Lane 1 – 18S rRNA (137bp), Lane 3 – ND1 (136bp), Lane 4 – H2 (147bp), Lane 5 – ND2 (136bp) and Lane 6 – COX I (136bp). Lane 2 is a DNA ladder where the first two bands are 100bp and 200bp respectively. qPCR products are free of contamination and are of the correct size.
Fig. 4.5 Melting curve analysis of qPCR products. Each product is free of impurities and/or non-specific amplification as shown by the single distinct peak above the threshold (horizontal blue line) and the melting temperature being close to the predicted value.

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>PREDICTED Tm</th>
<th>ACTUAL Tm</th>
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</thead>
<tbody>
<tr>
<td>18s rRNA</td>
<td>83.56</td>
<td>85.3</td>
</tr>
<tr>
<td>12S rRNA</td>
<td>77.6</td>
<td>81.8</td>
</tr>
<tr>
<td>H2</td>
<td>78.1</td>
<td>79.9</td>
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<tr>
<td>ND1</td>
<td>77.4</td>
<td>80.9</td>
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<tr>
<td>ND2</td>
<td>77.8</td>
<td>80.9</td>
</tr>
<tr>
<td>COX I</td>
<td>77.9</td>
<td>81.7</td>
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</table>
The qPCR itself yielded very interesting results (Fig. 4.6). It appears that the 3229.A mutation significantly lowers transcription termination in proportion to the mutation load, as seen by the ~50% (heteroplasmic lymphoblasts) to the ~150% (homoplasmic cybrids and muscle) increase in the genome-length H2 transcript in the proband compared to controls (the horizontal red line) when normalised to 18S rRNA levels. Similar results are obtained when normalising to the mitochondrial 12S rRNA. It must be noted that since we are measuring a fragment of the H2 transcript that does not include the 12S rRNA sequence, the results are a true reflection of changes in mtDNA expression relative to the 12S rRNA. It might be argued that the 3229.A mutation could affect the rate of transcription of either the H1 or H2 transcripts or both, leading to an erroneous measurement of downstream mRNA levels compared to 12S rRNA levels. No evidence of this was found as the 12S rRNA levels were relatively constant across control and proband samples.

It would be expected that an increase in H2 would lead to an increase in the mRNAs downstream of the transcription termination breakpoint. Although the proband muscle shows some increase in ND1 expression, the largest and most statistically significant increases in all downstream genes studied was found in proband lymphoblasts, regardless of whether the values are normalised to 18S or 12S rRNA. Interestingly, the homoplasmic cybrid shows no increase in any of the mt-mRNAs.

The results shown include two controls and were reproducible with different control cell lines.
Fig. 4.6 Results of qPCR using REST-RG. The 3229.A mutation causes a significant ($p \leq 0.05$) increase in various mtDNA transcripts in all the sample types studied. In tissues homoplasmic for the mutation, there is ~2 to 2.5-fold increase in the genome-length H2 transcript. Of the three sample types, lymphoblasts show the maximum increase in downstream mRNA levels. Similar results are obtained using either 18S rRNA or the mitochondrial 12S rRNA as reference genes. The red line indicates normal expression. Error bars indicate +/- SEM. Asterisks represent non-significant $p$-values.
4.2.2 Analysis of mRNA levels by Northern blotting

To confirm the qPCR results and to visualise the changes in proband mtDNA expression, I used the semi-quantitative traditional method of Northern blotting. The agarose gel (pre-transfer) and the nitrocellulose blot (post-transfer) were photographed to check for sample quality and efficiency of transfer (Fig. 4.7). There is no sample degradation and the ratio of the 28S and 18S rRNAs is close to 2, indicating that the RNA preparation was excellent. Given the limitations of capillary transfer, I still achieved a reasonably efficient transfer of the RNA onto the membrane. The results of probing the membrane with DIG-labeled DNA probes specific for COX I, and for 12S rRNA and 18S rRNA as loading controls, are shown in Fig. 4.8. After normalising to either reference gene and compared to the mean of two controls, the proband’s cybrids and lymphoblasts show an increase in COX I expression of ~10% and 35% respectively. Although this does not mimic the results obtained with qPCR, it confirms that the 3229.A mutation disrupts transcription termination and causes an increase in mt-mRNA levels.

Although I did not analyse the blots with probes for 16S rRNA or tRNA\textsuperscript{Leu(UUR)}, the 12S rRNA-probed blots showed neither a reduction in rRNA levels in the proband nor any evidence of any intermediate species such as the 19S RNA found in cells harbouring the A3302G mutation (Maniura-Weber et al. 2006) which established that the only species being measured by the qPCR was in fact the H2 transcript.
Fig. 4.7 Separation and transfer of total RNA. 10µg of total RNA from each sample was separated on a 1.5% formaldehyde-agarose gel and transferred onto nitrocellulose membranes by capillary transfer. The photograph shows that RNA samples are not degraded and that transfer has been reasonably efficient. The 28S (upper band) to 18S (lower band) rRNA ratios are all close to 2, indicating that the RNA preparation is of high quality. Lanes 1-2: cybrid controls; Lane 3: proband cybrid; Lanes 4-5: lymphoblast controls; Lane 6: proband lymphoblast.
Northern blot results. Blots were probed with DIG-labeled DNA probes specific for 18S or 12S rRNA (loading controls) or for COX I mRNA. The results are expressed as a ratio of the mRNA to the rRNA. When normalised to the nuclear-encoded 18S rRNA, proband’s cybrids and lymphoblasts show an increase in COX I mRNA levels of ~10% and 35% respectively compared to mean controls. There is also a decrease in the mitochondrial 12S rRNA:COX I mRNA ratio in proband compared to controls. Lanes 1-2:cybrid controls; Lane 3:proband cybrid; Lanes 4-5:lymphoblast controls; Lane 6: proband lymphoblast.
4.2.3 Immunoblot analysis of COX subunit levels

Since an increase in mRNA levels might lead to increased translation of a transcript, I decided to measure the levels of two mitochondrially-encoded subunits of COX (I and II). Since it was unlikely that the 3229.A mutation had any direct effect on nuclear-encoded mitochondrial proteins, I chose to use COX subunit IV as a reference protein. Using commercially available monoclonal antibodies to each of these subunits, I performed Western blotting, the results of which are seen in Fig. 4.9. Please note that in this figure alone, the proband lymphoblast sample is in Lane 4.

After normalising to COX IV and compared to the mean of two controls, the proband’s cybrids show an increase of ~11% and 45% in COX I and II levels respectively. His lymphoblasts show an increase of ~25% and 21% for the same subunits.

4.2.4 BN-PAGE analysis of mitochondrial holocomplexes

The possibility that an increase in mitochondrially-encoded respiratory chain subunit levels could trigger a corresponding increase in OXPHOS holocomplex levels led me to measure them by BN-PAGE (Fig. 4.10). Densitometric analysis of the Coomassie G250 levels by fluorescent laser scanning revealed no reproducible differences in mitochondrial holocomplex levels between the control and proband cells.
Fig. 4.9 Immunoblot results. Crude mitochondrial preparations from the proband and two controls for each cell type were solubilised with 2% SDS, separated on a 12.5% discontinuous polyacrylamide gel and transferred onto nylon membranes. The blots were probed with monoclonal antibodies to two mitochondrial-encoded subunits of COX (COX I and II) and one nuclear-encoded subunit (COX IV). Results are presented as a ratio of COX I or II to COX IV. There is an increase in the levels of the mitochondrially-encoded subunits in the proband as compared to controls. The increase in COX I levels loosely corresponds to the increase seen in COX I mRNA levels. Lanes 1-2: cybrid controls; Lane 3: proband cybrid; Lanes 4: proband lymphoblast; Lane 5-6: lymphoblast controls.
Fig. 4.10  Blue-Native PAGE analysis of mitochondrial holocomplex levels.
(a) Crude mitochondrial preparations from the proband and two controls for each cell type were solubilised with 1.5% dodecyl-maltoside and separated on a 4-16% native polyacrylamide gel. Individual bands were excised and identified using mass spectrometry. (b) Densitometry revealed no major differences in holocomplex levels between proband and controls (representative scan shown). Lanes 1 & 5:molecular weight marker; Lanes 2-3:cybrid controls; Lane 4:proband cybrid; Lanes 6-7:lymphoblast controls; Lane 8:proband lymphoblast.
4.3 Discussion

The basic transcription machinery of mitochondria has been characterised (Clayton 1984, 1991). Our understanding of these molecular events may be crucial in designing therapeutic solutions for diseases involving mitochondrial dysfunction (Larsson and Clayton 1995; Wallace 1999; Smeitink et al. 2001). One of the key elements underpinning mitochondrial biogenesis is transcription termination and point mutations such as the MELAS A3243G substitution located in the mTERF binding sequence have been known to cause defects in protein synthesis (Chomyn et al. 1992). However, the link between such mutations and the molecular events dependent on transcription termination has thus far been tenuous.

This study shows for the first time a mutation that significantly attenuates transcription termination, leading to an increase in the levels of various mtDNA transcripts. There is compelling evidence that a single base insertion at the critical 3’ end of the transcription termination breakpoint can disrupt the event, although the mechanism by which this happens is unclear. There appears to be a direct link between mutation load and the production of the genome-length H2 transcript, with homoplasmic cells and tissues showing a doubling in H2 levels, compared to the primary H1 transcript as represented by 12S rRNA levels. This is in stark contrast to earlier findings that the wild-type state shows a 15-60 fold excess of the two rRNAs as compared to the mt-mRNAs (Gelfand and Attardi 1981; Chomyn et al. 1992) or that in certain tRNA^{Leu(UUR)} mutations such as the A3302G substitution that affect transcription, the termination event is only disrupted to the point of producing increased levels of the 19S truncated
polycistron that contains the 16S rRNA, tRNA_{Leu(UUR)} and a portion of the ND1 gene (Maniura-Weber et al. 2006). Since the H2 transcript has been shown to be synthesised 25-fold less than the H1 transcript (Denslow et al. 1989; King and Attardi 1989; Kruse et al. 1989), the two-fold excess of H2 over H1 I observed in the proband cells are strikingly significant.

Another interesting finding was that the most significant increases in mt-mRNA levels were observed in the heteroplasmic lymphoblasts of the proband. Since Northern blotting did not reveal any intermediate RNA species, one can assume that this mutation does not impair post-transcriptional processing of the H2 polycistron. We would therefore expect to see a corresponding increase in the levels of the downstream mRNAs but this is clearly not the case with the homoplasmic cybrid cells from the proband. The reasons for this are unclear but may involve efficient RNA degradation and recycling pathways in the parent 143B206 osteosarcoma cell line. The proband’s muscle did show increased ND1 and ND2 levels but the paucity of muscle tissue precluded further analysis of mt-mRNA levels. Since the lymphoblasts were derived from the proband’s lymphocytes, the results suggest that his cells and tissues are not efficient in clearing the excess mitochondrial RNA produced by the 3229.A mutation, which might play a role in his disease phenotype.

Although the H1 transcript is synthesised at a far higher rate than the genome-length polycistron that yields the majority of the tRNAs and mRNAs in the mitochondrion, an earlier study has shown that the steady-state level of tRNA_{Val} derived from the H1 transcript is only 3-fold more than the levels of other tRNAs.
King and Attardi 1993). I have evidence that the downstream mRNA levels are elevated in the proband so it is safe to assume that tRNA levels would have also increased, although this was not studied. This hypothesis, in conjunction with the tRNA^{Leu(UUR)} folding studies, suggests that protein synthesis might be affected in the proband, with impaired translation being implicit based on the shift toward a less favourable energy state in the tRNA secondary structure. However, what I observed was that the excess mRNA, at least for COX I and II, appears to be translated normally, leading to an increase in the levels of these two subunits. Even though the mRNA:protein ratio *in vivo* need not be 1:1, there is a loose correspondence between the percentage increases in mRNA and protein levels in the proband.

As described in the introductory chapter of this thesis, the assembly of the OXPHOS enzymes is a complex process, involving the transport of the nuclear-encoded subunits, which from the vast majority of the holocomplexes, into the mitochondrion. It is possible that an increase in mtDNA-encoded subunit levels might trigger a corresponding increase in the levels of the nuclear-encoded ones, but I did not find any evidence of that by BN-PAGE. However, the sensitivity of the technique used might preclude measuring minute changes in holocomplex levels and a more detailed proteomic analysis is required to understand the molecular changes in the proband’s mitochondria. Any such changes would be likely to affect mitochondrial function, the study of which forms the basis of the next chapter.
Chapter V

Analysis of the Pathological Effect(s) of the

3229.A Mutation
5.1 Introduction

This chapter describes the results of experiments performed to determine whether the increased RNA levels caused by the 3229.A mutation produced changes in cellular homeostasis, by affecting OXPHOS metabolism, causing oxidative stress and/or by inducing apoptosis.

5.1.1 Measurement of respiratory chain activity

During OXPHOS, the mitochondrial respiratory chain (RC) enzymes transport electrons from reducing equivalents such as NADH or succinate to molecular oxygen, with the resulting large free energy loss being conserved by the oxidative phosphorylation of ADP into ATP (Lehninger 1982). Since these enzymes are composed of subunits encoded by both the nucleus and the mitochondrion, mutations in either can cause OXPHOS defects that are inherited autosomally or maternally respectively. Sporadic cases of OXPHOS metabolic deficit have also been reported and a number of mitochondrial mutations have been identified that impair respiratory chain function (Ruiz-Pesini et al. 2007).

Mitochondrial dysfunction is often tissue or organ-specific and could arise due to any or all of the following factors (Wallace 1992):

(i) tissue-specific isoforms of nuclear-encoded subunits of the RC enzymes
(ii) organ-specific energy requirements or regulation of electron flux, and,
(iii) the percentage of mtDNA heteroplasmy seen in different tissues.
Since OXPHOS defects in a cell are directly proportional to its mutation load (Wallace 1992), they can range from 0-100% of normal activity. Interestingly, the first report of a mitochondrial role in myopathy came from a patient with severe hypermetabolism (Luft et al. 1962), following which RC disorders have been mainly regarded as neuromuscular diseases (Rustin et al. 1994). In such cases, the investigation of OXPHOS deficit has been primarily carried out in skeletal muscle biopsy tissue, even though muscle may not be as involved as the CNS in mitochondrial disease (Trounce et al. 1996). Secondly, muscle fibres may show secondary OXPHOS defects that impede the study of primary ones, as seen for example in the T8993G substitution in Leigh disease in which the primary deficit is in ATP synthase activity. Skeletal muscle studies of Leigh disease patients have shown reduced OXPHOS function while somatic cell studies show normal RC function (Holt et al. 1990; Shoffner et al. 1992; Trounce et al. 1994). Transformed cell lines such as lymphoblasts and cybrids provide a powerful model system for OXPHOS study and are easy to grow and maintain (Trounce et al. 1994, 1996).

To investigate the effects of the 3229.A mutation on OXPHOS metabolism, I measured Complex I, Complex II + III and Complex IV activities in the proband and six controls each in muscle tissue, lymphoblasts and cybrids.

The measurement of mitochondrial ATP synthesis involves overcoming methodological problems such as the difficulty in delivering reaction substrates such as ADP to the mitochondrion, given the low permeability of the plasma membrane to such hydrophilic substrates. The isolation of sufficient coupled and intact mitochondria from cells to perform the assay is another issue and the use of whole cells is preferable. However, the cell membranes need to be permeabilised
with a suitable detergent to allow access to the reaction substrates. I chose to perform a fluorimetric assay that used firefly luciferase luminescence to measure ATP content in cybrids and lymphoblasts, with digitonin used to permeabilise cell membranes (Vives-Bauza et al. 2007). The reaction catalysed by luciferase is:

\[
\text{Luciferase} + \text{luciferin} + \text{ATP} \rightarrow \text{luciferase-luciferyl-AMP} + \text{PPi}
\]

\[
\text{Luciferase-luciferyl-AMP} + \text{O}_2 \rightarrow \text{luciferase} + \text{oxyluciferin} + \text{AMP} + \text{CO}_2 + \text{hv} \quad (562\text{nm})
\]

The reaction produces photons of wavelength 560 nm, whose intensity is proportional to the amount of substrates in the reaction mixture.

5.1.2 Measurement of oxidative stress

Mitochondria are involved in the production of reactive oxygen species (ROS) such as the superoxide and hydroxyl radicals, hydrogen peroxide and singlet oxygen which are the products of normal metabolism (Chance et al. 1979; Ambrosio et al. 1993) but can be potentially toxic when produced in large quantities, leading to tissue injury (Ambrosio et al. 1993). Mitochondria use about 98% of cellular oxygen to produce ATP and about 1-2% of it is converted by them into superoxide (Chance et al. 1979). They play a major role in lipid peroxidation and the production of semiquinone radicals (Chance et al. 1979; Richter 1992) and respiratory function declines with age due to long-term exposure to these free radicals (Richter 1992; Salganik et al. 1994).
Cellular DNA damage by ROS produces an established biomarker of oxidative stress, 8-hydroxy-2-deoxy guanosine (8-OHdG) (Floyd 1990; Spencer et al. 1995; Beckman and Ames 1997). The hydroxylation of guanosine can occur in response to changes in OXPHOS metabolism and increased levels of 8-OHdG have been associated with pathological conditions such as cancer and diabetes (Leinonen et al. 1997; Shen et al. 2007). The measurement of 8-OHdG levels in cells can be measured by using a commercially available competitive ELISA, in which 8-OHdG competes with a tracer (8-OHdG-acetylcholineesterase conjugate) for binding to a limited amount of 8-OHdG monoclonal antibody.

An earlier study in our laboratory had identified a novel T>C substitution at nt 1095 in the 12S rRNA of a patient with parkinsonism, deafness and neuropathy (Thyagarajan et al. 2000). Transmitochondrial cybrids from the patient showed evidence of increased oxidative stress in the form of reduced mitochondrial glutathione levels and a 10-fold increase in Caspase-3 activation (unpublished observations). Preliminary analysis of 8-OHdG levels in this proband cybrid, compared to two controls (Fig. 5.1 below) showed an ~10% increase in 8-OHdG levels in total cellular DNA. I decided to test the 3229.A proband and two controls (both cybrid and lymphoblasts) using this ELISA to see if the proband showed significant changes in 8-OHdG levels, which would then merit investigating glutathione and Caspase-3 levels.
Fig. 5.1 Preliminary analysis of 8-OHdG levels in a novel 12S rRNA mutation. Proband and control DNA samples were digested with Nuclease P1 and treated with alkaline phosphatase before being loaded on the ELISA plate according to manufacturer’s instructions. Each sample was run in triplicate. 8-OHdG levels were measured based on a calibration curve run on the same plate using serial dilutions of purified 8-OHdG. The proband (T1095C) shows an ~10% increase in 8-OHdG levels compared to the mean of two controls. Error bars are +/- 2SD.
5.1.3 Analysis of apoptotic effects of the 3229.A mutation

Mitochondria act as calcium ion sinks by transporting the ion from the cytosol for the regulation of dehydrogenases involved in the citric acid cycle, and accumulating it in high concentrations (Nicholls and Budd 2000; Nicholls 2005). The transport of calcium into and out of the mitochondrion is effected by specific proteins and increased Ca\(^{2+}\) loading causes the formation of a mitochondrial permeability transition pore (PTP) in the inner membrane (Norenberg and Rao 2007). This pore allows large solutes to enter the mitochondrial matrix, causing osmotic swelling of the mitochondrion which dissipates the proton gradient, stops ATP synthesis and initiates the release of apoptotic factors (Nicholls and Budd 2000; Norenberg and Rao 2007). Recent studies have implicated mitochondria in both apoptosis and necrosis (Kroemer and Reed 2000). Apoptotic outer membrane permeabilization involves the release of proteins which are normally confined to the intermembrane space of mitochondria, including cytochrome c, certain procaspases, adenylate kinase 2 and apoptosis-inducing factor (Kroemer and Reed 2000). Studies with recombinant pro-apoptotic members of the Bcl-2 family of protein have shown that they probably cause apoptosis through the formation of a PTP (Kroemer and Reed 2000; Shults 2004) and it appears that this event marks a point of no return in apoptosis (Petit et al. 1997).

A biochemical hallmark of apoptosis is the degradation of DNA into oligonucleosomal fragments that are typically 180-200 bp in size (Bortner et al. 1995). These are formed by the action of endonucleases on the linker region between histones and multiples of this monomeric fragment produce the typical ladder associated with apoptotic cells (Bortner et al. 1995; Paddenberg et al. 1995).
In order to study the possible apoptogenic effects of the 3229.A mutation, I decided to analyse total DNA from proband and control cell lines to see if DNA laddering was present in these samples. Camptothecin is an alkaloid drug known to induce apoptosis by binding to topoisomerase I (Ulukan and Swaan 2002) and I used it to test if the proband and control lymphoblasts and cybrids were susceptible to apoptogenic factors.

5.2 Results

5.2.1 Respiratory chain activity in the proband

Citrate synthase (CS) is a mitochondrial matrix marker enzyme whose specific activity is commonly used to normalise the measurement of OXPHOS activity in samples (Trounce et al. 1996). The proband lymphoblast and cybrid samples showed no significant change in CS activity as compared to 6 controls in case (Fig. 5.2) while his muscle showed a slightly elevated activity.

After normalising to CS activity to account for differences in mitochondrial number, proband muscle showed a ~12% increase in Complex I activity (Fig. 5.3) but no such increase was observed in his lymphoblasts or cybrids. Due to the paucity of muscle tissue, it was not possible to repeat the experiment to perform statistical analysis of these results.

None of the proband samples showed any significant changes in Complex II + III activity compared to controls (Fig. 5.4), although in general, it was observed that they were on the lower end of the control range.
The proband muscle showed elevated Complex IV specific activity (~10%) compared to controls but no such increase was seen in his cells (Fig. 5.5). Again, these results could not be analysed statistically.
Fig. 5.2 Citrate synthase (CS) specific activity in proband cells and tissues, compared to controls (n=6 in each case). The activity of CS, a mitochondrial matrix marker enzyme, was determined from the reduction of DTNB at 412nm at 30°C by 100µg of crude mitochondrial extracts from proband and control lymphoblast, cybrid and muscle samples. The absorbance change was monitored over 2 min and specific activity in units/mg protein calculated as 1 unit = nmol DTNB reduced/min/mg protein. Proband muscles show slightly elevated CS activity. Error bars indicate +/- 2SD.
Fig. 5.3 Complex I (CI) specific activity in proband cells and tissues, compared to controls (n=6 in each case). The nmol of NADH oxidized/min by crude mitochondrial extracts was monitored by absorbance changes at 340nm at 30°C for 3 min. Specific activity was calculated at nmol NADH oxidised/min/mg protein. Results are shown as a ratio of CI to CS activity to account for differences in mitochondrial number between samples. Proband muscle shows an ~12% increase in CI activity. Error bars indicate +/- 2SD.
Fig. 5.4 Complex II + Complex III (CII + CIII) specific activity in proband cells and tissues, compared to controls (n=6 in each case). The reduction of cytochrome-c by crude mitochondrial extracts that been exposed 5 freeze-thaw cycles in liquid nitrogen was assayed at 550nm at 30°C for 3 min. Specific activity calculated as units/mg protein with 1 unit being nmol of cytochrome c reduced/min/mg protein. Results are shown as a ratio of CII + CIII to CS activity to account for differences in mitochondrial number between samples. No significant changes were observed between proband and control samples. Error bars indicate +/- 2SD.
Fig. 5.5 Complex IV (CIV) specific activity in proband cells and tissues, compared to controls (n=6 in each case). CIV activity was measured by following the oxidation of reduced cytochrome-C at 550nm at 30°C for 5 min. Specific activity was calculated as a pseudo first order rate constant (k) and expressed as k/min/mg protein. Results are shown as a ratio of CIV to CS activity to account for differences in mitochondrial number between samples. Proband muscle shows an ~10% increase in CIV activity. Error bars indicate +/- 2SD.
The results of the luciferase-based assay of ATP synthase activity is shown in Fig. 5.6. Although both proband lymphoblasts and cybrids showed elevated ATP-synthase activity compared to 2 controls each, the results were not found to be statistically significant ($p>0.05$).

5.2.2 Measurement of oxidative stress in proband cells

There were no significant differences observed in 8-OHdG levels between proband and two controls each in lymphoblasts and cybrids (Fig. 5.7), indicating that the 3229.A mutation does not appear to increase the production of ROS and cause oxidative stress in vitro.

5.2.3 Analysis of apoptogenic effects of the 3229.A mutation

When total genomic DNA was extracted from proband and control lymphoblasts and cybrids and separated on a 1% agarose gel, there was no evidence of any DNA laddering, a hallmark of apoptotic cells (Fig. 5.8). When the same cells were treated with 4 µg/ml of Camptothecin A for 4h, apoptosis was induced in them, causing the formation of a DNA ladder (Fig. 5.9).
Fig. 5.6 Complex V (CV, ATP synthase) specific activity in proband cells, compared to controls (n=2 in each case). ATP synthase activity was measured by following the formation of AMP from ATP in a reaction catalysed by luciferase and measuring the resultant emission of yellow-green light at 550nm. After accounting for ATP synthesis from other sources, mitochondrial ATP synthase was measured as nmol ATP/min/mg protein. Proband lymphoblasts and cybrids both show elevated ATP synthase activity but not at statistically significant levels ($p>0.05$). Error bars indicate +/- 2SD.
Fig. 5.7  Measurement of 8-OHdG levels in proband and two controls. The levels of 8-OHdG, a known biomarker of oxidative stress-induced DNA damage, was measured using a commercially available ELISA kit. Total DNA was extracted from samples and exactly 3 µg of DNA was digested with Nuclease P1 followed by treatment with alkaline phosphatase. Two dilutions of the digested samples were loaded in triplicate on the ELISA plate with a set of standards run alongside. No significant differences in 8-OHdG levels were found between proband and controls. Results shown are an average of 3 ELISAs with the error bars showing +/- 2SD.
Fig. 5.8  Analysis of DNA laddering due to apoptosis in proband and controls cells. Total genomic DNA was extracted from cell samples and separated on a 1% agarose gel. No evidence of DNA laddering was found in any of the samples. Lane 1: molecular weight marker; Lane 2: positive control (U937 cells treated with 4 µg/ml Camptothecin A); Lanes 3-4: cybrid controls; Lane 5: proband cybrid; Lanes 6-7: lymphoblast controls; Lane 8: proband lymphoblast.
Fig. 5.9  Analysis of DNA laddering after induction of apoptosis in proband and controls cells. Cells were treated with 4 µg/ml Camptothecin A for 4h, following which total genomic DNA was extracted and separated as before. DNA laddering was seen in all samples, indicating that apoptosis could be induced in these cells. Lane 1: molecular weight marker; Lanes 2-3: cybrid controls; Lane 4: proband cybrid; Lanes 5-6: lymphoblast controls; Lane 7: proband lymphoblast.
5.3 Discussion

There appears to be a 10-12% elevation in Complex I and Complex IV activities in the proband’s muscle which could arise from a number of reasons. It is possible that an increase in mtDNA-encoded subunits of the respiratory chain complexes could lead to an increase in the levels of these proteins, in turn leading to increased biochemical activity. While this hypothesis needs to be tested in muscle samples, given that the mitochondrial subunit increases observed in proband lymphoblast and cybrid cells do not seem to cause any increases in holocomplex levels or in RC activity, it is unlikely that it is the predominant phenomenon in muscle.

Another possibility is that the increase in RC activity is in response to the higher load on the cellular transcription machinery imposed by attenuation of transcription termination and the subsequent need to clear the excess RNA produced. This might explain the elevation in ATP synthesis seen in proband cells, although the rise observed is not statistically significantly different from control cells. An electron leak across the RC gradient might also explain the observed increase in Complex I, IV and V activities in the proband and might merit further investigation.

Small changes in OXPHOS activity, such as the ones observed here, need to be interpreted with great caution. Among the pitfalls of spectrophotometric measurement of RC activity is that the data has been shown to not be normally distributed (Rustin et al. 1991), rendering mean and SD calculations as
meaningless. Studies have suggested that in order for a change in RC activity to be called significant, it might be required to demonstrate a difference of greater than 3SD (Rustin et al. 1994). The majority of mitochondrial disorders are characterised by a deficit in OXPHOS activity so the observed small increase could reflect methodological artifacts such as inconsistent freezing of muscle biopsy samples between proband and controls, or the fact that perfectly age-matched controls could not be obtained. Finally, the paucity of muscle tissues and the difficulty in obtaining them precluded repeat experimentation, so the observed results cannot be said to be absolute. It is important to note that once again, the homoplasmic cybrid model did not mimic biochemical events occurring in the proband’s homoplasmic tissues.

The role of mitochondria in apoptosis has been well characterised and mitochondrially-induced apoptosis is associated with neurodegeneration (Carrozzo et al. 2004). Susceptibility to apoptosis might be increased by the high levels of ROS generated by OXPHOS activity (Raha and Robinson 2001) and studies have shown that skin fibroblasts containing high levels of the NARP ATPase6 mutation show a huge induction of superoxide dismutase (SOD) activity, triggering apoptosis by overriding cellular antioxidant defenses (Geromel et al. 2001). It follows that increased transcription, translation or RC activity would lead to increases in ROS levels, causing oxidative stress and/or triggering apoptosis. No evidence was found that the 3229.A mutation induces ROS over-production, causing DNA damage or apoptosis. The cells were susceptible to chemically-induced apoptosis, suggesting that the mutation does not prevent such events either. However, it is important to keep in mind that the homoplasmic cybrid cells
showed no major increases in mt-mRNAs, other than the H2 transcript, nor did it show any increase in biochemical activity. Unless the oxidative stress and apoptosis studies are done with fresh proband muscle, it will not be possible to form a definite opinion on the possible apoptogenic effects of the 3229.A mutation.

In conclusion, this mutation appears to elevate RC activity in the proband but does not appear to disrupt cellular anti-oxidant defenses or induce apoptosis in vitro.
Chapter VI

General Discussion
6.1 Summary of findings

This study reports for the first time a novel adenine insertion at nt 3230 of the human mitochondrial genome that significantly disrupts transcription termination, leading to a 2-fold increase in the level of the genome-length mitochondrial H2 polycistron in the cells and tissues of a patient with a mitochondrial myopathy. There was a corresponding increase in his mature mitochondrial mRNAs, indicating that this mutation does not appear to affect post-transcriptional processing of the H2 transcript. In cell lines derived from the proband, an increase (correlating to increased mt-mRNA levels) in the levels of at least two mtDNA-encoded respiratory chain complex subunits was observed. OXPHOS activity (mainly Complexes I and IV) was elevated in the proband’s muscle but not in lymphoblast and cybrid cells, where the mutation does not seem to up-regulate respiratory chain activity and the mutant load does not directly correspond with any changes seen in such activity, a phenomenon which has been observed in other studies as well (Dunbar et al. 1995). However, both cell lines showed elevated ATP synthase activity, although not at statistically significant levels. No evidence of oxidative stress was found in these cell lines and the 3229.A mutation did not appear to have an apoptogenic effect.

These findings suggest that the 3229.A mutation interferes with mTERF binding at the H1 termination locus and are novel in several ways. To date, there have been no human mutations listed that cause over-expression of mtDNA or increase the translation of mtDNA-encoded OXPHOS subunits. Mitochondrial disease is normally associated with a deficit in OXPHOS metabolism. The proband has a
mitochondrial myopathy characterized by lifelong effort intolerance and myalgia, and rather than an OXPHOS deficit, appears to have elevated respiratory chain activity in tissues homoplasmic for the mutation. It can therefore be postulated that attenuation of transcription termination in the mitochondrial genome affects cellular homeostasis at several levels and is a novel mechanism of mitochondrial disease.

6.2 Pathophysiology of the 3229.A mutation

The etiology of the 3229.A mutation needs to be investigated. One of the earliest identified mt-tRNA\textsuperscript{Leu} mutations associated with a severe myopathy, the A3302G substitution at the 3’ end of the tRNA\textsuperscript{Leu}, was shown to cause a significant increase in an unprocessed mtRNA intermediate (RNA19) comprising the 16S rRNA, tRNA\textsuperscript{Leu} and ND1 (Bindoff \textit{et al.} 1993; Maniura-Weber \textit{et al.} 2006). Here disease pathology was attributed to improper processing of RNA19 and a subsequent drop in tRNA\textsuperscript{Leu} levels leading to impaired protein synthesis. It might be argued that the findings of this study reflect the presence of such an intermediate RNA species; however, the significantly higher levels of downstream genes such as ND2 and COX I, coupled with increased levels of COX I and II subunits, preclude such a possibility and suggest that it is the H2 transcript in its entirety that is being up-regulated. The enthalpy change in tRNA\textsuperscript{Leu} predicted by RNA-folding studies does not appear to affect mitochondrial protein synthesis.

The actual attenuation of transcription termination most likely occurs due to impaired binding of mTERF with its recognition sequence. It is possible that the
extra base insertion could structurally impede this binding but not completely abolish it, as evinced by the fact that, although significant statistically, there is only a doubling of the H2 transcript levels and not a larger change. Another possibility is that mTERF may still bind normally to its recognition sequence but its function is impaired by the conformational change in the mtDNA caused by the insertion.

6.2.1 RNA-mediated disease

A pathogenic gain of function has been attributed to untranslated RNAs in neurological disorders such as muscular dystrophy and spinocerebellar ataxia (Ranum and Cooper 2006; O'Rourke and Swanson 2009), with suggested mechanisms including the formation of toxic RNA structures and protein sequestration by the non-coding RNAs. Gain of function was suggested but has not been demonstrated in the A3302G myopathy.

The pathogenic RNAs associated with hereditary disease are invariably composed of expansions of microsatellite (2-6 bp) repeats. This study suggests that there may be a toxic gain of function at the RNA level in mitochondria harboring the 3229.A mutation; if proven, this will be a novel paradigm in mitochondrial genetics since it involves polycistronic coding sequences as well as mature mRNAs and tRNAs, rather than the non-coding repeats normally associated with RNA gain of function.

Without further detailed analysis, it is currently only possible to speculate about the actual toxicity of the over-expressed RNAs in the proband. One of the possible
scenarios is that the excess mRNA competes with normally expressed mRNAs for transcription factors as well as translation factors. It is also possible that there is a severe drain on the nucleotide pool in the cell to accommodate the excess mtDNA transcription, leading to a disruption of transcription in the nuclear genome. Finally, there could be a feedback-based increase in RNAse and protease levels within the cell in response to the need to process the over-abundant macromolecules produced as a result of this mutation, causing a pathogenic disruption of various cellular pathways. Interestingly, there was a comparable increase (~ 2 fold) in the H2 transcript in the homoplastic 3229.A cybrid and proband muscle but the cell line showed only marginal increases in downstream transcripts and no pathology. It is possible that the parent 143B206 cell line is able to efficiently degrade and recycle the increased levels of RNA with little or no deleterious effect. The possibility of the cell line processing the polycistron inefficiently is precluded by the lack of evidence of intermediate or truncated RNA species in either the control or the proband lines.

6.2.2 Protein gain of function

A gain of function has been attributed to protein-mediated toxicity in several dominantly inherited neurodegenerative disorders such as spinocerebellar ataxia types 1, 2 and 3, in which CAG expansions are translated into polyglutamine repeats (Ranum and Day 2004). In this case, there may be a protein gain of function in that the over-expressed mtDNA-encoded OXPHOS subunits may interfere with the assembly of respiratory chain holocomplexes, or even contribute to the assembly of highly unstable and pathogenic “super” complexes. As
mentioned above, cellular homeostasis could be disrupted by the need to degrade and recycle the excess protein.

### 6.3 Limitations of this study

The main limitation of this study was the difficulty in obtaining sufficient muscle tissue to examine directly disease mechanisms in the patient. For example, it is important to see if an increase in holocomplex levels is responsible for the observed elevation in proband muscle OXPHOS activity or if it is a response to cellular stress caused by over-expression of mtDNA. Although this study did not detect any significant differences in proband OXPHOS activity and holocomplex levels *in vitro*, the sensitivity of the assays used might not reflect very small but pathologically significant changes in the cells.

The other limitation was that even a homoplasmic 143B206-based cybrid was not found to be suitable to characterize this mutation since the changes in OXPHOS activity observed in the homoplasmic tissues of the proband could not be replicated in it. This might be a result of mtDNA expression regulation inherent to the parent ρ0 cell line, which has been observed in other studies tracking changes in mtDNA expression using 143B206-based cybrids (Hao *et al.* 1999; Swerdlow 2007). Secondly, if the hypothesis that the parent line is very efficient at clearing excess RNA is correct, then it is not a suitable model for studying a mutation whose primary effect is to disrupt transcription termination. It is important to note that any data obtained from non-isogenic cybrids should be interpreted carefully, given that molecular and biochemical activity may not be normally distributed in a range of controls. In this study, that limitation was partially overcome by the
fact that each cybrid control was isogenic to its lymphoblast counterpart, as was the proband’s. Even though there were no significant changes in the proband’s OXPHOS activities compared to controls, it would have been ideal to statistically analyse the distribution in the matched lymphoblast-cybrid pairs. However, the sample size and the fact that there was only patient to study precluded this.

The ideal scenario in the analysis of mitochondrial disease, especially with a novel mutation, is to be able to study the entire pedigree in order to match the genotype to possibly different phenotypes as well as understand the pathology of the mutation better. That was not possible in this case but hopefully will be achieved by future projects.

6.4 Future scope

These findings provide a platform for the development of several interesting research projects. They include, but are not restricted to, the following:

1. Analysis of the mitochondrial proteome to identify cellular factors that may be up-regulated due to the increase in mitochondrial mRNA and protein levels. The use of 2D gel electrophoresis followed by mass spectrometry may help identify level changes and/or modifications such as phosphorylation or glycosylation in cellular transcription and translation factors.

2. Analysis of the crystal structure of mutant OXPHOS complexes to determine whether unstable supercomplexes are being formed. Coupled with bioinformatics studies, it might be possible to identify which
mtDNA-encoded subunits contribute to less than favourable energy changes in holocomplex structure, if any.

3. Modelling and characterisation of mTERF interaction with mutated binding site to precisely determine the mechanism by which the 3229.A mutation interferes with transcription termination.

4. Development of different cybrid models to identify a more suitable one than the 143B206-based cybrid model for the study of the attenuation of transcription termination.

6.5 Publications arising out of work contained in this thesis

The following manuscripts are either in the process of submission or will be submitted soon. Asterisks indicate that the material was presented at a conference.


2. Slee M, Krupa M, Macardle P, **Raghupathi R**, Chataway T, Sims N and Thyagarajan D. The mitochondrial inner membrane is hyperpolarised in Primary Progressive Multiple Sclerosis. *


characteristics of a novel mtDNA deletion producing progressive external ophthalmoplegia associated with multiple sclerosis-like disease.

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