Molecular mechanisms in mitochondrial diseases

by

Hannah Rutherford Cock
Department of Clinical Neurosciences
Royal Free Hospital School of Medicine
University of London

A thesis submitted, in fulfilment, for the degree of
Doctor of Medicine

June 1996
Abstract

Despite the characterization of human mitochondrial DNA (mtDNA), many of the molecular mechanisms involved in the pathogenesis of mitochondrial diseases, in particular the role of nuclear genes, are poorly understood. Cultured cells from families with Leber's hereditary optic neuropathy (LHON), and from subjects with Hutchinson-Gilford progeria syndrome (HGPS), a clinical model of accelerated ageing, were studied.

Complex I activity was not decreased in 11,778 LHON lymphoblasts, or in 14,484 LHON fibroblasts, but a severe defect was confirmed in 3,460 LHON fibroblasts. However further characterization of the 3,460 fibroblasts demonstrated normal ATP synthesis in the presence of complex I linked substrates. This was not due to abnormal rotenone binding, inadequate sensitivity of the ATP assay, or a threshold effect. Analysis of specific complex I subunits, and mitochondrial protein synthesis in 3,460 fibroblasts were qualitatively normal. Fusion of enucleated 3,460 fibroblasts with $\rho^0$ cells was able to restore complex I enzyme activities to control levels, supporting a role for nuclear genes in the biochemical expression of this mutation. However the functional inconsistencies between the different mtDNA mutations, and the failure to distinguish clinically affected from unaffected individuals further complicates the debate on the role of mitochondrial dysfunction in the pathogenesis of LHON.

A decline in mitochondrial function with donor ageing was confirmed in cultured fibroblasts from healthy controls. All cell cultures had low levels of mtDNA bearing the "common" deletion. Fibroblasts from two subjects with HGPS exhibited significant defects in complexes II/III and IV, which were not observed in fusion cybrids generated from enucleated HGPS fibroblasts and $\rho^0$ cells. Studies on mtDNA and mitochondrially synthesized proteins in HGPS did not detect any qualitative abnormalities. It is proposed that mitochondrial dysfunction, secondary to a nuclear defect, may play a role in the pathogenesis of HGPS. This could have bearing on the findings in biological ageing.
Acknowledgements

There are many people I wish to thank for their help and support throughout my time at the Royal Free. Firstly I would like to thank the Medical Research Council who provided the funding that made it possible at all.

Everyone in the department helped to make my time there very enjoyable. I am especially grateful to Andrea and to Maria who taught me most of the techniques when I started, but thanks also to Lousie, Jan Willem, Roozina, Mei, Maggie, and Tracey for being around to answer questions and put me right, to Roger for his computer know-how, to John Muddle for the Image analysis, to Alan in Medical Engineering for the cover slips and various repairs, and to Mike for mycoplasma testing and a friendly ear!

To say thankyou to Mark Cooper conveys insufficient gratitute for his knowledge, experience, personal skills and support throughout - but Mark, thankyou. I am also especially indebted to Vin for advice, not always graciously recieved, and for being there.

Others I wish to thank for their friendship and support through these times include Sybl, Helen and Julian, Estelle, Fiona, Tristan, Helen and Jack - you know who you are.

Finally I do of course owe particular thanks to Tony Schapira, who took me on not knowing how much I had hated undergraduate biochemistry. That all changed very quickly. I could not have wished for better support, better company, or a better supervisor. Many thanks indeed.
Table of Contents

Part 1. Introduction

Chapter 1. Mitochondria
1.1 Mitochondrial genetics 14
1.2 The mitochondrial respiratory chain 17
1.3 Mitochondrial disease 26

Chapter 2. Leber's hereditary optic neuropathy (LHON)
2.1 Clinical features 33
2.2 Pathology in LHON 35
2.3 Historical theories in LHON 36
2.4 Mitochondrial DNA and LHON 36
2.5 Mitochondrial morphology in LHON 45
2.6 Mitochondrial function in LHON 46
2.7 X-linked/ nuclear genes in LHON 48
2.8 Auto-immunity in LHON 49
2.9 Questions remaining in LHON 50

Chapter 3. Ageing and Hutchinson-Gilford progeria syndrome (HGPS)
3.1 Features of normal ageing 52
3.2 Non-mitochondrial theories of ageing 53
Part 2. Methods

Chapter 4. Materials and methods
4.1 Clinical details and cell lines
4.2 Tissue Culture
4.3 DNA analysis
4.4 Mitochondrial preparations
4.5 Respiratory chain enzyme analysis
4.6 Measurement of ATP synthesis in permeabilized cells
4.7 Analysis of complex I proteins
4.8 Analysis of complex IV proteins
4.9 Mitochondrial in vitro translation studies on cultured fibroblasts
4.10 Presentation of results and discussion

Part 3: LHON results and discussion

Chapter 5. MtDNA analysis and preliminary MRC enzyme analysis in LHON
Introduction
5.1 Mitochondrial enrichment
5.2 11,778 LHON
5.3 14,484 LHON
5.4 3,460 LHON
5.5 Discussion: MtDNA and respiratory chain enzyme analysis in LHON
Chapter 6: Functional and structural characterization of complex I in 3,460 LHON fibroblasts

Introduction

6.1 ATP synthesis in 3,460 LHON fibroblasts
6.2 Rotenone sensitivity in 3,460 LHON fibroblasts
6.3 DPI sensitive NADH:CoQ1 reductase activity in 3,460 LHON fibroblasts
6.4 NADH:CoQ1 reductase enzyme kinetics
6.5 Mitochondrial protein studies in 3,460 LHON fibroblasts
6.6 Discussion: Functional and structural characterization of complex I in 3,460 LHON

Chapter 7: Nuclear genomic complementation in 3,460 LHON

Introduction

7.1 Characterization of the A549ρ0 cell line
7.2 Growth characteristics of fibroblasts with the 3,460 LHON mutation
7.3 Enucleation conditions and efficacy
7.4 DNA analysis of A549ρ0-fibroblast cybrids
7.5 MRC enzyme activities in 3,460-A549ρ0 fusion cybrids
7.6 Discussion: Nuclear genomic complementation in 3,460 LHON

Chapter 8. General discussion: mitochondrial abnormalities in the pathogenesis of LHON

8.1 Mitochondrial dysfunction as a pathogenetic factor in LHON
8.2 Pathogenetic factors in LHON unrelated to MRC dysfunction
8.3 Future work in the study of LHON
Part 4: Hutchinson-Gilford progeria syndrome and ageing: results and discussion

Chapter 9. Mitochondrial changes in ageing

Introduction 187
9.1 MtDNA in cultured fibroblasts from ageing donors 187
9.2 MRC enzyme analysis in cultured fibroblasts from ageing donors 188
9.3 Discussion: Mitochondrial changes in cultured fibroblasts from ageing donors 192

Chapter 10. Mitochondrial studies in HGPS

Introduction 201
10.1 Growth characteristics of HGPS fibroblasts 201
10.2 MtDNA in HGPS fibroblasts 202
10.3 MRC enzyme analysis in HGPS fibroblasts 202
10.4 COX western blots in HGPS 205
10.5 Mitochondrial translation studies in HGPS 206
10.6 Nuclear complementation of mitochondrial dysfunction in HGPS 206
10.7. Discussion: Mitochondria in HGPS 212

Chapter 11. Mitochondrial dysfunction in the pathogenesis of HGPS

11.1 General discussion: mitochondrial dysfunction in the pathogenesis of HGPS 218
11.2 Future work in the study of HGPS 221

References 223
Appendices 257
List of Tables:

Table 1. MtDNA mutations in LHON 37
Table 2. Disease models of ageing 67
Table 3. Primer details for PCR amplification of mtDNA fragments 82
Table 4. Restriction enzymes for identification of LHON mtDNA mutations 84
Table 5. Agarose gels for separation of restricted PCR products 85
Table 6. MRC enzyme activities in MEFs from 11,778 LHON lymphoblasts 104
Table 7. MRC enzyme activities in MEFs from 14,484 LHON fibroblasts 106
Table 8. MRC enzyme activities in MEFs from 3,460 LHON fibroblasts 109
Table 9. Complex I results from individual 3,460 subjects 110
Table 10. Densitometric assessment of % mutation in restricted PCR products from 3,460 fibroblast clones 113
Table 11. ATP production by control and 3,460 permeabilized fibroblasts 125
Table 12. DPI sensitive NADH:CoQ₁ reductase activity in MEFs from 3,460 fibroblasts 132
Table 13. MRC enzyme activities in MEFs from A549 cell lines 154
Table 14. Conditions yielding >90% enucleation for each of the cell lines fused 156
Table 15. MRC enzyme activities in MEFs from 3,460-A549ρ₀ cybrids and control-A549ρ₀ cybrids 163
Table 16. Correlation of MRC enzyme activities with age in fibroblast MEFs from controls aged 9-77 years 191
Table 17. Reports of MRC function in ageing 196
Table 18. MRC enzyme activities in MEFs from HGPS fibroblasts 204
Table 19. MRC enzyme activities in MEFs from HGPS-A549ρ₀ cybrids 211
List of Figures: page

Figure 1. Human mitochondrial DNA 15
Figure 2. The mitochondrial respiratory chain 18
Figure 3. Mae III restricted PCR products from lymphoblast cell lysates of one control and three 11,778 family members 102
Figure 4. Mbo I restricted PCR products from fibroblast cell lysates of one control and three 14,484 family members 105
Figure 5. HinI I restricted PCR products from fibroblast cell lysates of one control and three 3,460 family members 108
Figure 6. HinI I restricted PCR products from 3,460 fibroblast clones at p = 5 111
Figure 7. Standard densitometry curve of serial dilution's of the 630bp PCR product from 3,460 fibroblasts 112
Figure 8. HinI I restricted PCR products from selected 3,460 fibroblast clones at p = 10 113
Figure 9. Complex I activity in MEFs from fibroblast clones heteroplastic for the 3,460 LHON mutation 114
Figure 10. A sample ATP:fluorescence standard curve 123
Figure 11. Digitonin titration of a sample fibroblast line 124
Figure 12. Time course of ATP synthesis from a control fibroblast line 124
Figure 13. ATP production with complex I linked substrates in control and 3,460 LHON permeabilized fibroblasts 126
Figure 14. Rotenone inhibition of mitochondrial NADH:CoQ1 reductase activity, and of ATP synthesis with pyruvate and malate in control fibroblasts 128
Figure 15. Relationship between ATP synthesis and mitochondrial NADH:CoQ1 reductase activity in control fibroblasts 129
Figure 16. Rotenone sensitivity of mitochondrial NADH:CoQ1 reductase activity in MEFs from control and 3,460 fibroblasts 130
Figure 17. Rotenone inhibition of ATP production with pyruvate and malate in control and 3,460 LHON permeabilized fibroblasts 131
Figure 18. Kinetics of mitochondrial NADH:CoQ_10 reductase activity with respect to [NADH] 133
Figure 19. Western blot using a polyclonal antibody against the IP fraction of bovine complex I 134
Figure 20. Western blot using a polyclonal antibody against the 39kDa subunit of bovine complex I 135
Figure 21. Western blot using a polyclonal antibody against bovine complex I holo-enzyme 136
Figure 22. 35S labelled mitochondrially synthesized proteins from fibroblasts of one control and two 3,460 LHON subjects 137
Figure 23. 35S labelled mitochondrially synthesized proteins from fibroblasts of one control and three 3,460 LHON subjects 138
Figure 24. Growth curves of A549ρ^0 cells 151
Figure 25. PCR products... from cell lysates of control-A549ρ^0 cybrids and A549ρ^0 cells 153
Figure 26. Growth curves of control and 3,460 LHON fibroblasts 155
Figure 27. Poor (A) and >90% (B) enucleation of fibroblasts 157
Figure 28. Hinl I restricted PCR products from control-A549ρ^0 cybrid clones 159
Figure 29. Hinl I restricted PCR products from control-A549ρ^0 and III_2(clone 2)-A549ρ^0 cybrid clones 159
Figure 30. Hinl I restricted PCR products from control-A549ρ^0 and IV_1-A549ρ^0 cybrid clones 160
Figure 31. Hinl I restricted PCR products from high passage number A549ρ^0 cells, and 3,460-A549ρ^0 and control-A549ρ^0 cybrid clones 160
Figure 32. Genetic fingerprints using the multiloci probes 33.15 and 33.16 161
Figure 33. Complex I activity expressed as a CS ratio in MEFs from fibroblasts and A549$\rho^0$ cybrids

Figure 34. PCR products from control fibroblasts using primers for mtDNA with the common deletion

Figure 35. Xba I restricted and unrestricted PCR products from a patient known to possess mtDNA with the common deletion

Figure 36. The effect of donor age on MRC enzyme activities in MEFs from cultured fibroblasts of healthy donors

Figure 37. PCR products from HGPS and control fibroblasts using primers for mtDNA with the common deletion

Figure 38. MRC enzyme activities in MEFs from control and HGPS fibroblasts

Figure 39. Western blots using a cocktail of monoclonal antibodies directed against COX subunits I, II and IV

Figure 40. $^{35}$S labelled mitochondrially synthesized proteins from control and HGPS fibroblasts

Figure 41. PCR products from DP-A549$\rho^0$ cybrid clones

Figure 42. Alu I restricted and unrestricted PCR products from parental A549 cells, DP fibroblasts, and DP-A549$\rho^0$ cybrid clones

Figure 43. MRC enzyme activities in MEFs from control-A549$\rho^0$ and HGPS-A549$\rho^0$ cybrid clones
Abbreviations

AD(T)P  adenosine di-(tri-)phosphate

BMR  basal metabolic rate

bp  base pairs

BSA  bovine serum albumin

CNS  central nervous system

CoA  coenzyme A

CoQ₁  coenzyme Q-1

COX  cytochrome oxidase (complex IV)

CPEO  chronic progressive external ophthalmoplegia

CS  citrate synthase

CSF  cerebrospinal fluid

CT  computerized tomography

CVS  cardiovascular system

DMEM  Dulbecco's modified Eagles medium

DMSO  dimethyl sulphoxide

DNA  deoxyribonucleic acid

DPI  diphenyleneiodonium

DTNB  5,5'-dithio-bis-2-nitrobenzoic acid

EDTA  ethyleneglycol-diether tetraacetic acid

ELISA  enzyme-linked immunosorbent assay

FAD(H)  flavin-adenine dinucleotide, oxidized (reduced)

f.c.  final concentration

FMN  flavin mononucleotide

FP  flavoprotein

G6PDH  glucose-6-phosphate dehydrogenase

HGPS  Hutchinson-Gilford progeria syndrome

HP  hydrophobic

Hsp  heat shock protein

Ig  immunoglobulin

IP  iron protein

kDa  kilodaltons

KSS  Kearns-Sayre syndrome

LHON  Leber's Hereditary Optic Neuropathy

MEFs  mitochondrial enriched fractions

MELAS  mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes

MERRF  myoclonic epilepsy with ragged-red fibres

MIMyCa  maternally inherited myopathy and cardiomyopathy

MPP  1-methyl-4-phenlypyridinium
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
</tr>
<tr>
<td>MRC</td>
<td>mitochondrial respiratory chain</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>MRS</td>
<td>magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>MS</td>
<td>multiple sclerosis</td>
</tr>
<tr>
<td>mtDNA</td>
<td>mitochondrial DNA</td>
</tr>
<tr>
<td>mtTEFR</td>
<td>mitochondrial termination factor</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>mtTFA</td>
<td>mitochondrial transcription factor A</td>
</tr>
<tr>
<td>NAD(H)</td>
<td>nicotinamide adenine dinucleotide, oxidized (reduced)</td>
</tr>
<tr>
<td>NADP(H)</td>
<td>nicotinamide adenine dinucleotide phosphate, oxidized (reduced)</td>
</tr>
<tr>
<td>NARP</td>
<td>neurogenic weakness, ataxia and retinitis pigmentosa</td>
</tr>
<tr>
<td>NRFs</td>
<td>nuclear respiratory factors</td>
</tr>
<tr>
<td>OH8dG</td>
<td>8-hydroxy-deoxyguanidine</td>
</tr>
<tr>
<td>p</td>
<td>passage number</td>
</tr>
<tr>
<td>( \rho^0 )</td>
<td>rho-zero (mtDNA-less)</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson's disease</td>
</tr>
<tr>
<td>PDL</td>
<td>population doublings</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
</tr>
<tr>
<td>R.cyt.c</td>
<td>reduced cytochrome c</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RRFs</td>
<td>ragged-red fibres</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SCcR</td>
<td>succinate:cytochrome c reductase (complexes II/III)</td>
</tr>
<tr>
<td>TAE</td>
<td>tris-acetate-EDTA</td>
</tr>
<tr>
<td>TBE</td>
<td>tris-borate-EDTA</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloracetic acid</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer ribonucleic acid</td>
</tr>
<tr>
<td>v/v</td>
<td>volume for volume</td>
</tr>
<tr>
<td>w/v</td>
<td>weight for volume</td>
</tr>
</tbody>
</table>
Part 1: Introduction

Chapter 1. Mitochondria

The field of mitochondrial genetics has expanded exponentially since the characterization of human mitochondrial DNA in 1981 (Anderson et al. 1981), particularly in respect to the role of mitochondria in human disease. However many features of mitochondrial disease remain unexplained, particularly an understanding of the molecular mechanisms responsible for the wide diversity of clinical phenotypes resulting from a smaller range of defined mutations and biochemical defects. More recently information regarding the role of nuclear genes in mitochondrial function and regulation has emerged, and may provide some answers. The aim of this thesis is to further characterize the molecular mechanisms involved in the expression of a much studied mitochondrial disease - Leber's Hereditary Optic Neuropathy (LHON). In addition similar work has been carried out on a clinical model of accelerated ageing, Hutchinson-Gilford progeria syndrome (HGPS), in which for the first time in this condition a mitochondrial defect was identified.

1.1 Mitochondrial Genetics

Human mitochondrial DNA (mtDNA) is a closed circular molecule of 16,659 base pairs. Its complete sequence and functional assignment of the genes have been understood for some time (Anderson et al. 1981; Chomyn et al. 1985). MtDNA (Figure 1) contains no introns but consists almost entirely of genes encoding 22 tRNA species, a 16s and 12s rRNA, and 13 polypeptides all of which contribute to the mitochondrial respiratory chain (MRC). Our understanding of mtDNA replication and transcription is still expanding, but certainly involves nuclear encoded proteins. A gene free region referred to as the D-loop (displacement loop) contains the origin of heavy(H)-strand replication (OH) from where nuclear encoded DNA-polymerase γ, imported from the cytoplasm, initiates H-strand synthesis and proceeds clockwise around the
Figure 1. Human mitochondrial DNA encompasses 16,569 base pairs. Numbering starts at the origin of heavy chain replication (O_H) and proceeds anticlockwise. Genes on the light chain are identified inside the circle. The promoter regions for the heavy and light strands are labelled P_H and P_L respectively. Complex I subunits are labelled ND1 - ND6; cyt b = cytochrome b (complex III); complex IV subunits are labelled COI - COIII; tRNA genes are indicated by the letter of their cognate amino acid as follows: P = tRNA proline, T = tRNA threonine, E = tRNA glutamic acid, L = tRNA leucine(CUN), S = tRNA serine, H = tRNA histidine, R = tRNA arginine, G = tRNA glycine, K = tRNA lysine, D = tRNA aspartic acid, Y = tRNA tyrosine, N = tRNA asparagine, C = tRNA cysteine, A = tRNA alanine, W = tRNA tryptophan, M = tRNA methionine, Q = tRNA glutamine, I - tRNA isoleucine, L = tRNA leucine (UUR) V = tRNA valine, F = tRNA phenylalanine.
circle to a second origin of replication for the light strand ($O_L$; Clayton, 1992).
MtDNA replication occurs independent of the cell cycle, and as each cell may have several hundred copies of mtDNA any mutation which arises may only be present in a proportion of the total cellular complement. This can lead to a cell having 2 (or more) populations of mtDNA - normal and mutated, a condition known as heteroplasmy. As the cell divides the mutant and normal mtDNA molecules are randomly distributed to the daughter cells. Heteroplasmy may persist, but this replicative segregation can cause drift of the genotype ultimately towards either pure mutant or pure wild-type mtDNA (homoplasmy). Clearly mutations which interfere with mtDNA replication would not be expected to survive this process but other factors influencing the direction of such drift are poorly understood. Mitochondrial transcription also starts within the D-loop from 2 adjacent promoters, one for each strand ($P_H$ and $P_L$), resulting in one giant poly-transcript containing all the mRNAs. A nuclear product, mitochondrial transcription factor A (mtTFA), has been shown to bind to these promoters and stimulate transcription in vitro. MtTFA is also required for efficient replication of mtDNA (Attardi, 1993). In addition nuclear respiratory factors (NRFs) have been identified with recognition sites both in nuclear genes with products contributing to the MRC (Virbasius and Scarpulla, 1994), the mtRNase P gene and in a proximal promoter of the mtTFA gene. As such NRFs have the potential to communicate nuclear regulatory events to the mitochondrial replication/transcription machinery, and could also feedback cytoplasmic signals relating to the cellular energy state to the nucleus.

Once transcription has taken place many changes to the transcript are necessary before translation can occur. All of the enzymes involved are nuclear encoded, along with the majority of subunits in the final enzyme complexes. Specific sequences proximal to the promoters of such nuclear genes, for example the ATP synthase β gene, seem to act as positive or negative transcriptional factors. In vitro studies have shown that binding factors to these regions, named OXBOX and REBOX, respond to intracellular signals and
1.2 The mitochondrial respiratory chain

The mitochondrial respiratory chain (MRC) represents the means by which cellular ATP is generated through oxidative phosphorylation (Figure 2). It consists of 5 multi-subunit enzyme complexes (I - V) situated on the inner mitochondrial membrane (Darley-Usmar et al. 1994). MtDNA encodes 7 of the so far 41 identified subunits of complex I (NADH:ubiquinone oxidoreductase), cytochrome b of complex III (ubiquinone:cytochrome c oxidoreductase), 3 subunits (COX I, II and III) of 13 in complex IV (cytochrome c oxidase), and 2 of 13 subunits in complex V (ATPase). The remaining subunits and all of complex II (succinate:ubiquinone oxidoreductase) are nuclearly encoded. Also within the inner membrane are electron carriers such as the quinoid structures FMN, FAD and ubiquinone, and transition metal complexes in the form of iron-sulphur clusters and heme, and cytochrome c.
Figure 2: The mitochondrial respiratory chain, situated on the inner mitochondrial membrane depicted here in dark grey.

The enzyme complexes are shown as pale shaded areas, the lipid-soluble electron transfer shuttle ubiquinone as Q, and the water soluble transfer protein cytochrome c as C. Electrons from matrix substrates NADH and FADH₂ enter at complexes I and II, and electrons pass along the chain (\( \rightarrow \text{e} \)) to the final electron acceptor, oxygen. Coupled vectorial proton translocation (\( \uparrow \text{H}^+ \)) creates an electrochemical gradient which is utilized by the final complex, V, to synthesize ATP. Further details are given in the text.
Complexes I - IV make up the electron transport chain, oxidising NADH and FADH$_2$ and passing the electrons to the terminal acceptor oxygen, which is reduced to water. At complexes I, III and IV oxidative energy is conserved via coupled vectorial proton translocation and the creation of an electrochemical gradient across the inner mitochondrial membrane. This is utilized by complex V to synthesize ATP. Up to the level of cytochrome c (complex III) the system is reversible, but the final step from cytochrome a$_3$ to oxygen is irreversible which displaces the equilibrium in the direction of ATP synthesis. Diagramatically the stoichiometry is represented as 1:1, but in vivo it is estimated to be complex I (1): complex II (2): complex III (3): cytochrome c, complex IV and complex V (6 each).

1.2.1 Biosynthesis of the MRC

Following co-ordinate expression of nuclear and mitochondrial coding genes as discussed in Chapter 1.1, nuclear encoded proteins, synthesized on cytoplasmic ribosomes, then need to be transported into the mitochondria and directed to the appropriate internal compartment for biosynthesis of the complete enzyme complexes (reviewed by Kübrich et al. 1995). Initially cytosolic proteins interact with specific outer membrane proteins, directed by targeting sequences present on the precursor molecules, and then present to a general insertion pore made up of several subunits. The subsequent translocation across the inter-membranous space and the mitochondrial inner membrane into the mitochondrial matrix has been much studied. An initial driving energy dependant on the membrane potential presents the amino-terminal to the matrix space where, after cleavage of the precursor sequence, mitochondrial heat shock protein 70 (Hsp70) binds to it and pulls the protein across the membrane. The protein is then transferred to Hsp60 which mediates ATP-dependant refolding. Hsp60 is one of a group of molecular chaperones ("chaperonins") and may also play a role in the assembly of the final multi-subunit complexes as mutant yeast cells lacking Hsp60 synthesize nuclear
encoded mitochondrial proteins normally and successfully import them into the organelle, but they fail to assemble into the oligomeric enzyme complex (Cheng et al. 1989). A case of a patient with a generalized mitochondrial respiratory chain defect in association with very low levels of Hsp60 has been reported (Huckriede and Agsteribbe, 1994). This is not a feature of other mitochondrial diseases and implies that the primary defect affects Hsp60 with secondary consequences on MRC function.

1.2.2 Complex I

1.2.2.1 Basic structure

NADH:ubiquinone oxidoreductase is the most complex of all the MRC proteins. It catalyses electron transfer from NADH to quinone analogues, NAD and ferricyanide. Ubiquinone reduction, though not that of FeCN, is linked to proton translocation and can be inhibited by rotenone, piericidin A, 1-methyl,4-phenylpyridinium (MPP+), barbiturates and mercurials (Walker, 1992).

On the basis of chaotropic separation the polypeptides of complex I can be separated into three groups: about 70% of the total protein is hydrophobic (HP fraction), and the 30% remaining water soluble protein can be further subdivided into an iron-protein fraction (IP) and a flavoprotein fraction (FP). Purification and identification of its composite subunits has been difficult, but complex I probably contains over 40 polypeptide subunits as based on a combination of denaturing and 2-dimensional gel-electrophoresis, cloning and sequencing methods. The total molecular mass would be over 800kDa (Walker, 1992; Darley-Usmar et al. 1994).

The FP fraction includes the NADH dehydrogenase complex with the NADH binding site probably localized to its largest subunit (51kDa). 24 and 10kDa subunits have also been consistently identified by immunoprecipitaion and Western blotting. Binuclear and tetranuclear Fe-S clusters are thought to be related to the 24 and 51kDa subunits. All three FP subunits are exposed to the matrix side of the membrane based on ELISA (Han et al. 1988) and
functional (Krishnamoorthy and Hinkle, 1988) studies on purified protein, intact mitochondria and membrane preparations or submitochondrial particles.

The IP fraction consists of 6 major polypeptides of 75, 49, 30, 18, 15 and 13kDa each. No enzymatic activity has been related to any of them. There are 2 binuclear Fe-S clusters associated with the largest subunit, and a binuclear and tetra-nuclear Fe-S cluster with each of the 49, 30 and 13kDa subunits. Again all are exposed to the matrix side of the membrane, though the 75, 49 and 30kDa may be transmembranous. The HP fraction contains an indeterminate number of remaining polypeptides and probably all of the mitochondrially encoded subunits (Ohnishi, 1993).

The exact topography of the complete complex is unclear. It is thought to exist as a dimer and as at least some of the IP fraction proteins span the membrane yet contain no hydrophobic sequences, it is likely that the domains within the HP fraction surround the IP and FP fractions and protect them from the lipid bilayer.

1.2.2.2 Binding sites and inhibitors

Much attention has been focused on the binding sites of complex I inhibitors particularly as many of the biochemical assays of mitochondrial complex I function rely on detecting specifically inhibitable rates of NADH dehydrogenase, for example rotenone sensitive NADH:ubiquinone reductase (Ragan et al. 1987). Furthermore endogenous or exogenous complex I inhibitors may play a role in some disease mechanisms (Schapira et al. 1990a). Both rotenone and piericidin are inherently hydrophobic and will bind non-specifically to lipid membranes, but this non-specific binding can be displaced by washing with albumin. Titration curves relating to specific binding are sigmoidal suggesting dual co-operative binding sites may exist. Furthermore inhibition by most MPP+ derivatives and by rotenone is biphasic with a partial immediate effect followed by a slower onset complete inhibition. The former may represent an external matrix exposed site with a secondary hydrophilic site
as complete inhibition by hydrophilic inhibitors is enhanced in the presence of an ion-pairing carrier such as tetraphenylboron (Singer and Ramsay, 1994). This would act by facilitating access to the binding sites buried in the lipid membrane (Murphy et al. 1995). The 33kDa ND1 subunit is implicated in rotenone binding (Singer and Ramsay, 1994). This binding is also proportional to NADH:ubiquinone reductase activity implying that ND1 is involved in electron transfer. Functionally the major inhibition site for rotenone, MPP and piericidin is situated between the highest potential Fe-S cluster and ubiquinone. It was initially suggested that this might be through direct displacement of ubiquinone, but it is now more likely that these inhibitors are less direct in their action, acting perhaps though a conformational change in the enzyme unfavourable for electron flux to ubiquinone (Singer and Ramsay, 1994). A mutant human cell line lacking the ND4 gene product has been described in which the other mtDNA products also fail to assemble with a complete loss of NADH:ubiquione oxidoreductase activity, though nuclear encoded proteins assemble normally, implying that the ND4 subunit is also essential for complex I activity (Hofhaus and Attardi, 1993). Functional roles for other mitochondrially encoded subunits cannot be excluded.

1.2.3 Complex II

Succinate:ubiquinone oxidoreductase is the smallest and simplest of the MRC enzymes and is entirely nuclear encoded. It's major component is succinate dehydrogenase which is the membrane bound enzyme found in all aerobic organisms as part of the citric acid cycle. This is made up of 2 hydrophilic proteins of 70 and 27.5kDa each, and also contains covalently bound FAD, and Fe-S complexes. In addition to oxidising succinate the complex catalyses the transfer of reducing equivalents to ubiquinone or dyes such as ferricyanide, but it is not involved in proton translocation. The remaining 2 subunits lack specific catalytic activity but are essential for the physiological functioning of the complex. The 15.5kDa subunit has several hydrophobic
sequences long enough to traverse the inner mitochondrial membrane and it is likely that this and the smallest 13.5kDa subunit perform a structural role in the final complex (Darley-Usmar et al. 1994).

1.2.4 Complex III

Ubiquinol:cytochrome c oxidoreductase catalyses electron transfer from dihydroubiquinone to cytochrome c, coupled to transmembranous proton translocation. Situated in the middle of the MRC is accepts electrons from complexes I and II as well as those derived from fatty acid oxidation, fed into the ubiquinone pool via electron-transferring flavoproteins. There at least 11 subunits in mammalian complex III and 4 redox centres: cytochromes b$_{566}$ and b$_{562}$, cytochrome c and a binuclear Fe-S centre known as the Rieske centre. All the subunits are nuclear encoded except for cytochrome b, a 35kDa membrane spanning hydrophobic protein which contains both b hemes. The c heme has been localized to subunit IV, and the Rieske iron protein is subunit V. Other subunits, numbered according to molecular weight, lack redox centres or catalytic activity. I and II are known as the core proteins, and probably play a role in the assembly of the enzyme. Complex III activity is inhibited by antimycin A which binds near the high potential b heme (Darley-Usmar et al. 1994).

1.2.5 Complex IV

Cytochrome oxidase (COX) is the terminal complex of the mitochondrial respiratory chain and catalyses the oxidation of cytochrome c, the reduction of oxygen and the translocation of protons. In mammals there are 13 subunits revealed on SDS-PAGE of which the largest, COX I, II and III are encoded by mitochondrial DNA and constitute the catalytic core of the complex. The prosthetic groups involved in electron transfer have been localized to COX I and II and are generally accepted as consisting of two hemes, a and a$_3$, and two copper atoms. COX III is thought to be involved in proton translocation.
COX is inhibited by carbon monoxide, cyanide and azide all of which form a complex with $a_3$, especially in its reduced state (Capaldi et al. 1995).

1.2.5.1 Nuclear encoded COX subunits and tissue specific isoforms

The sequence of all the bovine heart COX subunits is known, and the nomenclature of Kadenbach and Mearle (1981) is most widely used. This labels the nuclear encoded products as IV, Va, Vb, VIa, VIb, VIc, VIIa, VIIb, VIIc and VIII. However the function of nuclear encoded products is less well defined. Work on yeast mutants lacking specific nuclear subunits implies they are needed for the correct assembly of the final complex, so may be of primary structural importance or play a role in directing protein importation and intramitochondrial sorting (Capaldi, 1990). In addition cytochrome oxidase is well situated as a potential regulator of mitochondrial respiratory chain activity in which nuclear encoded subunits may play a role. In keeping with this a number of tissue specific isoforms of nuclear subunits are known to exist in mammals. In beef there are at least two forms of subunits VIa, VIIa and VIII - a heart form (H) predominating in cardiac and skeletal muscle, and a liver (L) form in brain, liver and kidney. This varies between species and in humans only the L form of subunit VIII has been found. Of further interest is the identification of isoforms for subunit VIIc which differ only in the N-terminal extension/pre-sequence of the precursor form, having identical mature polypeptides. This subunit may also have different foetal and adult isoforms, implying participation in developmental regulation of this complex (Bonne et al. 1995).

1.2.6 Complex V

The ATP synthase complex is responsible for ATP synthesis from ADP and inorganic phosphate driven by the protonic energy derived from electron transfer through complexes I to IV. The are 2 major domains: $F_1$ contains the catalytic centre and is water soluble, protruding into the mitochondrial matrix; $F_0$
is hydrophobic, embedded in the inner membrane and is concerned with proton translocation.

The eukaryotic enzyme consists of 14 or more polypeptides of which 2, ATPase 6 and 8, are encoded by mtDNA. Both of these belong to the membrane fraction. Functional roles for many subunits have been defined. Tissue specific isoforms for the F₁-α subunit have been identified in bovine and human proteins which might have important implications for the tissue expression of clinical defects (Darley-Usmar et al. 1994).

1.2.7 Inter-mitochondrial communication

Whilst an individual mitochondrion might be expected to function according to the mtDNA and proteins that it alone possesses there is accumulating evidence that all the mitochondria within a cell effectively function as a single unit. Using specific staining techniques for mtDNA and mitochondrial proteins Hayashi et al. (1994b) showed that only six hours after fusion of stained cytoplasts with a mtDNA-less HeLa cell line the donor mtDNA was distributed throughout all the parental mitochondria. Furthermore fusion of two distinct cell lines each containing large deletions of mtDNA has been shown to result in inter-mitochondrial complementation of respiratory chain function (Davidson et al. 1995). Both the original cell lines had severe defects in MRC function and mitochondrial protein synthesis. The fusion cybrid contained both types of deleted mtDNA which between them provided uninterrupted copies of each mtDNA gene, and both MRC function and protein synthesis levels were normal. Some would extrapolate this evidence to suggest that the individual organelles we see on electron micrographs are merely slices through a single larger multil-lobulated mitochondrion in each cell, but this has not yet been proven.
1.3 Mitochondria and disease

Living human tissues are dependent on ATP production via oxidative metabolism, in turn reliant on the integrity of the MRC. Abnormalities anywhere in this sequence can hence result in disease. Before discussing LHON and ageing in more detail I shall first review some broad principles that can be learnt from other mitochondrial diseases.

Mitochondrial disorders may be inborn or acquired, and can be classified according to their genetic basis, biochemical expression or clinical phenotype. With our increased understanding of molecular genetics a classification strategy on this basis is being more widely used. Even so the picture is complicated by the fact that identical genetic and/or biochemical defects may manifest very different phenotypes clinically, and similarly the same clinical picture may be produced by a number of different mutations. A detailed review of mitochondrial disease is outside the scope of this thesis, but the basic categories will be mentioned in order to convey the complexities of studying mitochondrial disorders.

1.3.1 Primary mtDNA mutations

Mitochondria and hence mtDNA, due to their position in the cytoplasm of the ova and not in the head of the spermatozoa, are maternally inherited (Giles et al. 1980). Thus those diseases associated with primary mtDNA mutations also follow maternal inheritance in familial cases, though many are sporadic. All the progeny of a mother homoplasmic for a given mutation will have identical genotypes. However if the ovum (mother) is heteroplasmic, mostly the case with pathogenic mtDNA mutations, the proportions of wild-type and mutant mtDNA in the developing fetus are unpredictable and may vary not only between subsequent offspring, but also between tissues in a given individual. This clearly complicates predictions not only in respect to phenotype, but also to genotype.
Numerous mutations arising within the mitochondrial genome have now been described, and mtDNA does seem more susceptible to damage. Factors which contribute to this include the relative paucity of "redundant" DNA within the mitochondrial genome and its lack of histones and efficient repair mechanisms (Clayton et al. 1974). Combined with its proximity to the most prolific producer of free radicals within the cell in the form of the MRC (Chance et al. 1979), this makes mtDNA particularly susceptible to damage. It is also possible, given that at least in vitro dysfunction of the MRC leads to further free radical production (Cleeter et al. 1992), that existing mtDNA mutations which interfere with normal MRC function initiate a vicious cycle of ever increasing damage to nearby structures, including mtDNA itself.

1.3.1.1 MtDNA Rearrangements

Heteroplasmic rearrangements of mtDNA, predominantly deletions, are the most frequently encountered mtDNA mutations and are mostly found in patients with CPEO (chronic progressive external ophthalmoplegia) or KSS (Kearns-Sayre syndrome; Holt et al. 1988). They have also been reported in cardiomyopathy (Hattori et al. 1991), diabetes mellitus with deafness (Ballinger et al. 1992) and Pearson's marrow-pancreas syndrome (Rötig et al. 1990). Such cases are usually sporadic and the deletion is typically flanked by direct repeats, possibly arising through a slip replication model (Mita et al. 1990). More recently it has become apparent that many patients with deletions also have small duplications of mtDNA within the D-loop (Brockington et al. 1993). The duplicated mtDNA includes the heavy-strand and light-strand promoters and so results in a partial excess of replication machinery which could increase the risk of replication slippage though generation of displaced heavy strands, and lead to the formation of deleted mtDNA molecules. Although the disease causing deletions are not usually inherited per se, duplications may be maternally transmitted and be a transient intermediate in the formation of
deletions (Poulton and Holt, 1994). Duplications alone, ranging from a few bases to several kilo bases, may also cause disease (Poulton et al. 1989).

A 4977bp deletion spanning complex I and IV genes as well as intervening tRNAs is found in 30-40% of cases (Harding, 1993), though over 130 types of deletions ranging from 1.3 to 7.6Kb have been reported in muscle. There is no clear correlation between the site and size of the deletion and its biochemical phenotype, but nearly all deletions include tRNA's so might be expected to affect translation of additional coding genes outside the deleted region. Deficiencies of all the MRC enzymes is commonly seen (Moraes et al. 1989a).

The tissue distribution of deleted mtDNA clearly influences phenotype as the same deletion easily detectable in the blood of patients with Pearson's is typically absent in the blood of KSS patients, but present in high amounts in muscle. Furthermore at least two cases who have survived infantile Pearson's have gone on to later develop KSS with the appropriate shift in their tissue distribution of deleted mtDNA (McShane et al. 1991).

That deletions in KSS and CPEO are rarely detectable in blood, and not maintained in tissue culture has long been recognized (Moraes et al. 1989a). It has been suggested that in tissues with low cell replication rates but a high oxidative demand such as brain and muscle, high mitochondrial turnover with preferential replication of the smaller deleted mtDNA molecules, might account for the accumulation of deleted mtDNA. In rapidly dividing cells the biochemical disadvantage conferred by the deletion would slow growth and over several passages cells containing normal mtDNA come to predominate. This could account for the recovery usually seen in Pearson's syndrome where both the deletion and a major clinical problem primarily involve haemopoietic cells (Bourgeron et al. 1994).
1.3.1.2 MtDNA point mutations

Missense mutations in coding genes for MRC subunits have been found in LHON which will be discussed in more detail in Chapter 2, in NARP (neurogenic muscle weakness, ataxia and retinitis pigmentosa; Holt et al. 1990) and in Leigh's syndrome (Santorelli et al. 1993). A mutation causing a T to C, or T to G transition at position 8993 of the ATPase subunit 6 gene which appears to inhibit ATP production by blocking the proton channel of the ATPase, has been clearly implicated both of these last conditions. A high mutant load results in the fatal multisystemic infantile Leigh's syndrome, whereas when the proportion of mutated mtDNA falls below ~90% the more benign NARP phenotype is exhibited. With lower percentages still a peripheral retinopathy may be the only manifestation. All phenotypes may appear in the same family (Ortiz et al. 1993), and it is one of the few diseases where there is reasonably good correlation between mutant load and clinical presentation.

Point mutations within tRNA genes not surprisingly result in a widespread severe disruption to mitochondrial protein synthesis. Almost invariably these are heteroplasmic and associated with severe biochemical defects. As a group they account for some of the most diverse clinical pictures, with considerable variation in expression between individuals. Typically they result in severe mitochondrial myopathies hallmarked by "ragged-red" skeletal muscle fibres on Gomori trichrome staining, and the accumulation of structurally abnormal mitochondria on electron microscopy. The major disease entities causally associated with tRNA mutations are MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes; Goto et al. 1990), MERRF (myoclonic epilepsy with ragged-red fibres; Shoffner et al. 1990), and MiMyCa (Maternally inherited myopathy and cardiomyopathy; Mariotti et al. 1994). The biochemical pathogenicity of the MELAS 3243bp mutation has been demonstrated by transfer of mutant mitochondria into a control human mtDNA-less (ρ0) cells where the defects in mitochondrial protein synthesis and MRC function (complexes I, III and IV) persisted (Chomyn et al. 1991), though the
precise molecular mechanisms, particularly accounting for the varied clinical pictures seen, remain unexplained. As for deletions, mutant load and tissue distribution have some influence (de Vries et al. 1994).

Point mutations in ribosomal RNA mitochondrial genes are a more recently described phenomena, having been clearly associated with non-syndromic deafness (Prezant et al. 1993). The Arab-Israeli family in question are of particular interest as the inheritance pattern conformed best to a "two-hit" model involving both mtDNA and nuclear genomes. In addition the clinical phenotype is very focal in nature though the rRNA mutation was widely distributed including in blood. This inferred role for nuclear gene involvement has parallels with the clinical features of LHON as I shall go on to discuss, however to date there are no diseases in which pathogenic mutations in both nuclear and mitochondrial genomes have been characterized.

1.3.1.3 Nuclear-driven mutations

As the mitochondrial genome is so small and has been completely sequenced identifying mutations within it has been a relatively easy task. However given that the majority of MRC subunits are nuclear-encoded and with our increasing understanding of nuclear regulatory factors more genomic mutations causing mitochondrial disease can be expected to emerge. There are reports showing abnormalities of nuclear-encoded proteins in a few rare cases: deficient complex I subunits (Schapira et al. 1988), deficient complex III Rieske Fe-S protein (Schapira et al. 1990c) and multiple mitochondrial enzyme deficiencies with a deficiency of Hsp60 (Agsteribbe et al. 1993). However to date there is only a single report of a nuclear mutation in association with a mitochondrial disease (Bourgeron et al. 1995). This was found in two siblings, of consanguineous parents, who presented with Leigh's syndrome and complex II deficiency. A point mutation in a highly conserved flavoprotein subunit gene on chromosome 5 was homozygous in both affected children, heterozygous in both parents and not found in 120 controls.
It is also clear that in some cases rearrangements of mtDNA may be secondary to nuclear defects. There are several families described who demonstrate autosomal dominant inheritance of multiple deletions of mtDNA (Zeviani, 1992). The deletions commonly all arise within the D-loop, but differ in size and frequency within and between family members. This pattern suggests a nuclear defect which either increases an intrinsic propensity of mtDNA to undergo rearrangement, or reduces the organelles' ability to detect and eliminate deleted mtDNA. Defects in candidate genes have not yet been identified, but have been localized by linkage analysis to chromosome 10q in a Finnish family (Suomalainen et al. 1995). This localization excludes various candidate genes including several involved in counteracting oxidative stress, and although the gene for mtTFA is found on chromosome 10q sequencing in this family did not detect any mutation.

Finally in a rare fatal infantile disorder where the major problem is a reduced number of copies of mtDNA in each cell, known as mtDNA depletion (Moraes et al. 1991), fusion studies using $\rho^0$ cells have demonstrated that the defect is nuclear in origin (Bodnar et al. 1993). A deficiency of mtTFA has been associated with mtDNA depletion (Poulton et al. 1994) though whether this is the pathogenic event or a secondary marker is not clear. Pedigree analysis suggests autosomal inheritance, certainly dominant in one family where 3 affected infants had the same father but 2 separate mothers (Ricci et al. 1992).

1.3.1.4 Acquired MRC dysfunction

In addition to mutations of the mtDNA, the protein components of the MRC and the lipid membranes in which they sit may themselves be directly damaged by non-genetic events. Their close proximity to a potent source of free radicals is one possibility, supported by the high levels of antioxidants naturally found in mitochondria. In addition a significant number of naturally occurring and synthetic toxins have been shown to be specific inhibitors of the MRC. Perhaps the most famous of these is MPTP (1-methyl-4-phenyl-
1,2,3,6, tetrahydropyridine), a by-product of attempts to make a recreational synthetic opiate whose injection in a group of drug users led to a parkinsonian syndrome (Ballard et al. 1985). The active metabolite MPP+ (1-methyl,4-phenylpyridinium) is actively taken up into mitochondria and acts via specific inhibition of complex I of the MRC, binding at a site involving both nuclear and mitochondrial encoded subunits. With complex I inhibition a parallel fall in cellular ATP levels is seen, ultimately causing cell death. This effect can be attenuated by free radical scavengers. Free radicals are produced when MPP+ interacts with the MRC and themselves further inhibit complex I suggesting a self-perpetuating cycle of complex I inhibition and oxidative damage (Tipton and Singer, 1993). In susceptible individuals similar environmental toxins may be implicated in the pathogenesis of neurodegenerative disease, especially idiopathic Parkinson's disease in which there is a wealth of data implicating complex I dysfunction and oxidative stress contributing to cell death in the substantia nigra (Schapira, 1994c), though their precise role in pathogenesis remains to be clarified.

There is also a precedent for mtDNA mutations predisposing to environmental toxic damage. The 1555 rRNA mtDNA mutation associated with inherited deafness has also been implicated as a susceptibility factor for aminoglycoside toxicity: aminoglycosides act on bacterial rRNA, and in the Far East where they are widely used even therapeutic doses are commonly associated with deafness. Two large pedigree studies demonstrated that this antibiotic-induced deafness was maternally inherited, and subsequently the same rRNA mutation has been seen in these families (Prezant et al. 1993).

Finally in all cases disease develops on a background of the normal ageing process which to some extent may also represent acquired mitochondrial dysfunction and accumulation of mtDNA damage as I shall discuss in Part 3. Interactions between inherited mitochondrial genes, environmental factors and the passage of time must also play a role in the evolution of primary mitochondrial diseases.
Chapter 2. Leber's Hereditary Optic Neuropathy

2.1 Clinical features

LHON is recognized as the commonest cause of isolated blindness in young men with an estimated incidence of 1 in 50,000. The ratio of males to females amongst those clinically affected has probably been overestimated in the past but with molecular diagnosis now available the current literature estimate is 3:1 (Riordan-Eva and Harding, 1995).

Typically LHON presents as a subacute painless visual loss with a peripapillary telangiectatic microangiopathy on fundoscopy in the early stages (Nikoskelainen et al. 1983), later followed by non-specific optic atrophy. Milder forms may be seen in symptomatically unaffected individuals (Nikoskelainen et al. 1982). In most cases the onset is in the late teens and early twenties, though cases have been described at both extremes of age (Newman et al. 1991). In some cases acuity is lost from both eyes simultaneously, but in most the loss is sequential with an interim period of anything from days to years. Recovery is variable, and to some extent may be linked to the underlying mtDNA genotype as I will later discuss, but most affected individuals remain visually handicapped for life.

Visual evoked potentials (VEPs) are sometimes normal, and if abnormal differ from those characteristic of for instance multiple sclerosis, suggesting both demyelination and loss of nerve fibres (Carroll and Mastalgia, 1979). Asymptomatic and presymptomatic family members may also show minor changes on fundoscopy (Nikoskelainen et al. 1982), or have abnormal VEPs (Nikoskelainen et al. 1977), but neither feature has sufficient specificity or sensitivity to be of diagnostic use in atypical cases.

Prior to mtDNA analysis the diagnosis was a purely clinical one based on a characteristic features and a positive family history with strictly maternal inheritance (Seedorf, 1985), however sporadic cases are now being increasingly recognized (Weiner et al. 1993).
2.1.2 LHON and other CNS disease

There are many case reports of families who have the typical features of LHON and other CNS disease. Although early reports (Ferguson and Critchley, 1928) may be complicated by the difficulty in accurate diagnosis, even with the advent of mtDNA confirmation this association is clear. Most common are reports of LHON in the context of often clinically definite multiple sclerosis (MS) (Harding et al. 1992), although it may be difficult in these cases to know which disease is causing the visual loss. Screening MS patients for LHON mtDNA mutations have so far failed to show any definite association (Nishimura et al. 1995), except possibly in a small subgroup with early severe visual symptoms (Kellar-Wood et al. 1994). Severe encephalomyelopathy (Wallace, 1970), myelopathy (Wilson, 1963), peripheral neuropathy (Johns et al. 1993b), cerebellar ataxia (Funakawa et al. 1995) and dystonia (Jun et al. 1994a) have all been clearly described. In the more recent of these studies the diagnosis of LHON has been confirmed by molecular genetic analysis, but to what extent these reports represent chance associations is unclear. Mitochondrial mutations are more commonly associated with widespread disease and in some families the LHON and the CNS disease are clearly inherited together, so it seems likely that more than coincidence is operating. Nikoskelainen et al. (1995) reported neurological abnormalities in 27 of 46 affected LHON patients (59%) from 24 families with either the 3,460 or 11,778 mutations. However this was a selected and uncontrolled series and many of the abnormalities were minor (e.g. benign essential tremor) or had other potential contributory factors (e.g. alcohol abuse). The larger study by Riordan-Eva et al. (1995) found evidence of more widespread neurological abnormalities in only 7 of 107 affected patients and alcohol was thought to be the major contributor to 2 of these. The overall incidence of other neurological disease in LHON is probably thus rather low, and there is at least one report showing normal brain MRIs in genetically confirmed LHON (Kermode et al. 1989).
2.1.3 LHON and cardiac disease

Early clinical studies supported an excess of cardiac conduction abnormalities in association with LHON (Nikoskelainen et al. 1985) but most of the literature preceded molecular diagnosis. The only paper to address this question since has found no such association in the 49 LHON pedigrees studied (Newman et al. 1991). There is an 11,778 American family with a prolonged Q-T interval (Ortiz et al. 1992), but this may not be causally associated with the LHON mutation.

2.2 Pathology

Initial reports suggesting widespread abnormalities of brain pathology at post mortem in LHON almost certainly include other diseases and are hence difficult to interpret. Not surprisingly as LHON is predominantly a disease affecting otherwise healthy young people histopathological reports are sadly lacking. If one concentrates on the optic pathways of clinically typical patients findings are fairly consistent and tissue specific: there is a loss of ganglion cells in the retina; the optic nerve shows a severe loss of myelin and some reduction in nerve fibre diameter and number. This axonopathy extends to the optic tracts and lateral geniculate bodies, but the occipital cortex is normal (Hume Adams et al. 1966; Wilson, 1963; Kwittken and Barest, 1958). Similar optic tract findings were reported by Kerrison et al. (1995) in an 81 year old woman from the Queensland family possessing both the 14,484 and 4,160bp mutations. Electron-dense, double membrane-bound, calcium containing inclusions were also demonstrated in retinal ganglion cells in this case. Some of these inclusions appeared to displace rudimentary cristae, and as they were not present in age matched control eyes the authors concluded that they might represent calcified mitochondrial inclusions resulting from mitochondrial dysfunction. Confirmation of these findings in other molecularly confirmed LHON subjects is awaited.
2.3 Historical theories

As optic atrophy is an occasional consequence of various deficiency / toxic states much of the initial LHON research tried to identify detoxification pathway defects in LHON patients, particularly concerning cyanide metabolism and smoking. However results were limited to isolated case reports and small series e.g. Cagianut et al. (1981) and provide no conclusive evidence.

It had long been recognized that LHON though clearly familial could not be accounted for by Mendelian genetics and some reports correctly hypothesized an aetiological role for unidentified cytoplasmic factors (Imai and Moriwaki, 1936). The 1970 report by Wallace came very close to current thinking in suggesting both a vertically transmitted agent and nuclear genetics might be involved, though at that time hypothesized an infective agent plus polygenically determined variability in resistance. The first mention of possible mitochondrial inheritance was in 1972 (Erikson), but it was not until the characterization of human mitochondrial DNA in the 1980's that this idea could be readily pursued.

2.4 LHON and mtDNA mutations

Since the first report in 1988 (Wallace et al.) LHON has been associated with at least 18 mtDNA mutations (Table 1). The widespread use of simple laboratory techniques to identify these mutations in blood from suspected cases has eased the diagnosis of LHON and also revealed that up to 50% of patients may lack a characteristic family history, and would not previously have been diagnosed on clinical grounds alone (Riordan-Eva and Harding, 1995). All are missense point mutations mostly in genes encoding complex I subunits. Neutral mtDNA variants are commonplace particularly between ethnic groups, thus in LHON and other mitochondrial diseases criteria have been set by several authors for considering a mutation as pathogenetic (Howell, 1994). Strictly applied the mutation should be found only in association with disease and never in controls of varying phylogenetic backgrounds; it should not
Table 1. MtDNA mutations in LHON.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Pathogenic class</th>
<th>Gene</th>
<th>Nucleotide change</th>
<th>αα change</th>
<th>αα conservation</th>
<th>found in controls</th>
<th>references</th>
</tr>
</thead>
<tbody>
<tr>
<td>11,778</td>
<td>1°</td>
<td>ND4</td>
<td>G to A</td>
<td>R to H</td>
<td>H</td>
<td>no</td>
<td>Wallace (1988); Singh (1989)</td>
</tr>
<tr>
<td>3,460</td>
<td>1°</td>
<td>ND1</td>
<td>G to A</td>
<td>A to T</td>
<td>M</td>
<td>no</td>
<td>Huoponen (1991); Howell (1991a)</td>
</tr>
<tr>
<td>14,484</td>
<td>1°</td>
<td>ND6</td>
<td>T to C</td>
<td>M to V</td>
<td>M</td>
<td>no</td>
<td>Johns (1992a); Mackey (1992)</td>
</tr>
<tr>
<td>14,459</td>
<td>?</td>
<td>ND6</td>
<td>G to A</td>
<td>A to V</td>
<td>M</td>
<td>no</td>
<td>Jun (1994a)</td>
</tr>
<tr>
<td>4,160</td>
<td>?</td>
<td>ND1</td>
<td>T to C</td>
<td>L to P</td>
<td>H</td>
<td>no</td>
<td>Howell (1991b)</td>
</tr>
<tr>
<td>4136</td>
<td>2°</td>
<td>ND1</td>
<td>A to G</td>
<td>C to Y</td>
<td>M</td>
<td>no</td>
<td>Howell (1991b)</td>
</tr>
<tr>
<td>13078</td>
<td>2°</td>
<td>ND5</td>
<td>G to A</td>
<td>A to T</td>
<td>M</td>
<td>yes</td>
<td>Johns &amp; Berman (1991); Brown (1992b)</td>
</tr>
<tr>
<td>4917</td>
<td>2°</td>
<td>ND2</td>
<td>A to G</td>
<td>D to N</td>
<td>H</td>
<td>yes</td>
<td>Johns &amp; Berman (1991; Cornelissen (1993))</td>
</tr>
<tr>
<td>4216</td>
<td>2°</td>
<td>ND1</td>
<td>T to C</td>
<td>Y to H</td>
<td>L</td>
<td>yes</td>
<td>Johns &amp; Berman (1991); Gerbitz (1992)</td>
</tr>
<tr>
<td>15,257</td>
<td>2°</td>
<td>cyt.b</td>
<td>G to A</td>
<td>D to N</td>
<td>H</td>
<td>yes</td>
<td>Johns &amp; Neufeld(1991); Brown (1992b)</td>
</tr>
<tr>
<td>15,812</td>
<td>2°</td>
<td>cyt.b</td>
<td>G to A</td>
<td>V to M</td>
<td>M</td>
<td>yes</td>
<td>Johns &amp; Neufeld (1991); Brown (1992b)</td>
</tr>
<tr>
<td>5244</td>
<td>2°</td>
<td>ND2</td>
<td>G to A</td>
<td>G to S</td>
<td>H</td>
<td>no</td>
<td>Brown (1992b)</td>
</tr>
<tr>
<td>7444</td>
<td>2°</td>
<td>COX I</td>
<td>G to A</td>
<td>Term. to K</td>
<td>-</td>
<td>yes</td>
<td>Brown (1992c)</td>
</tr>
<tr>
<td>3394</td>
<td>2°</td>
<td>ND1</td>
<td>T to C</td>
<td>Y to H</td>
<td>H</td>
<td>yes</td>
<td>Johns (1992a)</td>
</tr>
<tr>
<td>13730</td>
<td>2°</td>
<td>ND5</td>
<td>G to A</td>
<td>G to E</td>
<td>M</td>
<td>no</td>
<td>Howell (1993a)</td>
</tr>
<tr>
<td>9,438</td>
<td>2°</td>
<td>COX III</td>
<td>G to A</td>
<td>G to S</td>
<td>H</td>
<td>yes</td>
<td>Johns and Neufeld (1993)</td>
</tr>
<tr>
<td>9,804</td>
<td>2°</td>
<td>COX III</td>
<td>G to A</td>
<td>A to T</td>
<td>H</td>
<td>no</td>
<td>Johns and Neufeld (1993)</td>
</tr>
<tr>
<td>9101</td>
<td>2°</td>
<td>ATPase 6</td>
<td>T to C</td>
<td>L to T</td>
<td>L</td>
<td>no</td>
<td>Lamminen (1995)</td>
</tr>
</tbody>
</table>

1° = primary; 2° = secondary; cyt.b=cytochrome b; H - highly conserved; M - moderately conserved; L = low conservation. αα = amino acids, represented by their conventional letter symbols.
coexist with other known pathogenic mutations and it should change a highly conserved amino acid; finally it should be found in a heteroplasmic state as heteroplasmy does not seem to be a feature of harmless polymorphisms. In the case of LHON this last condition is interpreted as heteroplasmy in at least some people from affected families, as the majority of LHON patients, affected or not, are in fact homoplasmic. This may reflect the fact that LHON generally does not affect either life-span or reproductive capacity, so the tendency for homoplasmic individuals to be selected against does not apply as it might in more deleterious mitochondrial diseases.

Some would also propose that alteration in function should be demonstrable, and this is true in most mitochondrial disease. However as will become apparent functional changes in LHON are not clearly defined. Three mtDNA mutations at positions 3,460, 11,778 and 14,484 are universally accepted as "primary" mutations in that they satisfy most of the above criteria. Further mutations at 14,459 and 4,160 are associated with additional CNS disease and await clarification. The remaining are classified as "secondary" affecting less conserved areas, often coexisting with each other or the primary mutations, and sometimes found in controls.

2.4.1 11,778bp LHON mutation

This G to A mutation at position 11,778 converting an arginine to histidine in the ND4 subunit of complex I was first reported in 4 LHON families of varied ethnicity in 1988 (Wallace et al.), since when many confirmatory reports have followed from Japanese (Yoneda et al. 1989), American (Singh et al. 1989), Australian (Johns and Berman, 1991) and European families (Holt et al. 1989). This mutation accounts for 50% - 80% of all LHON families and as such is the most common (Harding et al. 1995).

In the original pedigrees 80% of clinically affected individuals were male (Newman et al. 1991), with a mean age at onset of 28 years (range 8-60). A more recent and larger study (Harding et al. 1995) shows a male:female ratio of
only 3.7:1 which is more likely to be representative. This report also took into account that onset may occur as late as 62 years. In both reports virtually all patients suffered bilateral symmetrical visual loss, sequential in 50% of cases with an average delay between eyes of 1 to 4 months. In over 98% visual acuity declined over a period of weeks to less than 20/200 and only 4% showed any degree of significant recovery.

2.4.2 3,460bp LHON mutation

This was the second mtDNA mutation to be found in LHON families (Huoponen et al. 1991); The G to A mutation converts an alanine to a threonine in the ND1 subunit of complex I. As with the previous case this is a highly conserved amino acid in an evolutionarily constrained polypeptide domain and has not been found in any control populations. It accounts for 10 -15% of LHON families. While most of the clinical parameters are indistinguishable from those of the other primary mutations, the likelihood of visual recovery is greater (20%) than for the 11,778 mutation, and disease penetrance seems higher with more affected family members in these pedigrees (78% vs 43%) (Johns et al. 1992b).

2.4.3 14,484bp LHON mutation

When this mutation was first documented in a Queensland LHON family (Howell et al. 1991b) it was deemed insignificant. The 14,484 T to C mutation causes a valine substitution in place of methionine in the ND6 subunit of complex I and although not found in controls this site is only moderately conserved. However subsequently the same mutation has been described in many independent LHON probands and their families (Johns et al. 1992a; Mackey and Howell, 1992), and is clearly associated like the other primary mutations with a high degree of disease penetrance, and seems sufficient to result in disease. Clinical features are similar though the typical acute fundal changes may be lacking, the average age of onset is younger (19 years), and there is a better prognosis in terms of visual recovery (37% - 50%) particularly if
the onset age is below 20 years (Johns et al. 1993a). The 14,484 mutation is often associated with secondary mutations so may be less deleterious alone than the other primary mutations (Brown et al. 1994).

2.4.4 Leber's "plus" MtDNA mutations

2.4.4.1 4,160bp mutation

The Queensland family first described by Wallace et al. in 1970 display an unusually severe form of the disease, and debate as to the responsible mutation(s) remains. Given the striking clinical features some have doubted whether it is LHON at all (Bateman, 1994), but there is no doubt that the ocular features in the many individuals examined conform to those expected of LHON, as does the inheritance with all 20 adult males in the female line being affected, and 19 of the 30 female descendants from a total pedigree of 101 in five generations. Some individuals displayed encephalomyelopathy, fatal in several cases, which segregated with the LHON. Following the identification of a severe complex I defect in 1989 (Parker et al. 1989) sequencing of the mitochondrial complex I genes in this pedigree identified many polymorphisms, including the 14,484 mutation as discussed above (Howell et al. 1991b), and a T to C mutation at position 4,160. This substitutes a proline for leucine in a highly conserved region of ND1, was not found in 18 controls and segregated with clinical disease. It was thus considered to be of primary pathogenetic significance in this family, though the coexistence of the 14,484 mutation complicates this interpretation. The biochemical features of the 14,484 mutation have not been reported, but might shed light on which of these mutations is responsible at least for the biochemical defect in this family.

2.4.4.2 14,459bp mutation

Another mutation likely to be confirmed as primary is that at position 14,459, a G to A missense mutation changing a moderately conserved alanine to valine in ND6 (Jun et al. 1994a). This has been reported in a 4 generation
Hispanic family of whom 2 members have classical LHON, and 1 has LHON and a neurodegenerative disorder characterized by early onset dystonia with bilateral basal ganglia lesions. A further 8 members have only the neurodegenerative condition. This heteroplasmic mutation was found in all maternal relatives but not in 349 controls from varied ethnic backgrounds. A severe complex I defect was subsequently also found in the patients (Jun et al. 1994b). The same mutation and biochemical phenotype have since been found in two further independent pedigrees with different phylogenetic backgrounds (Shoffner et al. 1995), however it has not been found in an English family with a similar phenotype (personal communication, Prof.A.E.Harding, Institute of Neurology, London).

2.4.5 Secondary LHON mutations

The number of other missense mutations identified in LHON families is ever increasing. It is generally thought that these "secondary" mutations are insufficient alone to result in disease, but may do so in combination with one another, or may influence the penetrance of the primary mutations. They are less common overall in LHON families than the primary mutations, and several are also found in low frequencies in control populations. They have no clearly defined detrimental effect on biochemical function, though data is very limited in this respect. Only the more common or controversial mutations are discussed here. Details of all those so far identified are listed in Table 1.

2.4.5.1 13,708 LHON mutation

This has largely been found in association with 11,778 LHON (Johns and Berman, 1991), identified in up to 25% of such cases but has no clear effect on disease penetrance in these families (Gerbitz et al. 1992). It has also been reported with the 14,484 mutation (Johns et al. 1992a). It's prevalence in control populations is around 5% (Brown et al. 1992a), thus whilst it appears
more frequently in LHON families it alters only a moderately conserved amino acid, and may represent only a common Northern European polymorphism.

2.4.5.2 15,257 and 15,812 LHON mutations

The 15,257 mutation was first reported in patients lacking any other primary mutations and itself considered of major pathogenetic significance (Johns and Neufeld, 1991). Seven of the 8 cases also had the 13,708 mutation, and 4 of 8 a further cytochrome b mutation at 15,812. Its link to LHON was confirmed in other reports (Brown et al. 1992b). Initial information suggested a better visual prognosis, though a higher incidence of CNS and peripheral nerve disease but a larger study from the Netherlands, in pedigrees with each of the primary mutations, revealed no influence on clinical outcome (Oostra et al. 1994b). There are usually other secondary mutations present, and it is also found in 0.3% of controls and has been relegated to a secondary classification (Howell et al. 1993b).

The presence of the 15,812 mutation may have a detrimental effect clinically, though numbers are small (Johns et al. 1993b).

2.4.5.3 COX LHON mutations

Point mutations at positions 9,438 and 9,804 have been identified in probands with typical LHON lacking other known LHON mtDNA mutations (Johns and Neufeld, 1993). Both change highly conserved amino acids and were not found in the 400 controls used for this study. They were hence proposed as new primary mutations. However it has since been shown that though the frequency of the 9,438 mutation is less than 0.05% in non-African populations, it may be up to 18% in Africans with no evidence of LHON (Howell, 1994). Further data on the 9,804 mutation is awaited, but also likely to be no more than a polymorphism. This example highlights the difficulties in drawing conclusions from isolated studies in LHON, and the need to include controls from multiple ethnic backgrounds before assigning pathogenicity.
2.4.5.4. Summary of secondary mutations

The presence of secondary mutations may contribute to the clinical picture in a minority of LHON cases. Some 80-90% of LHON patients have one of the primary mutations. Aiming to assess the role of these less significant mutations, whilst our understanding of the primary pathogenetic mechanisms is so poor, is unlikely to be of immediate value. Identification of secondary mutations cannot be used to make a diagnosis of LHON, so routine screening is probably not justified.

2.4.6 Disease penetrance in LHON

That LHON is not a simple hereditary condition will already be apparent. We can now identify primary mutations with ease by a blood test (Hammans et al. 1991), but making clinical predictions based on such a result is difficult, with unpredictable disease penetrance even in the presence of a homoplasmic primary mutation. A homoplasmic male 11,778 patient has only ~50% chance of going blind, falling to ~15% if female (Riordan-Eva and Harding, 1995); Several factors may influence penetrance, many as yet unidentified.

2.4.6.1 Heteroplasmy

Most LHON families are homoplasmic for their mtDNA mutations but heteroplasmy has been documented in 11,778 (Holt et al. 1989), 14,484 and 3,460 families (Harding et al. 1995). Several reports suggest a link between mutant load and the likelihood of developing clinical LHON (Holt et al. 1989; Zhu et al. 1992) with only those with >95% mutation clinically affected though these are both small studies. The largest study to address this question (Smith et al. 1993) looked at 75 patients and 101 unaffected relatives with the 11,778 mutation of whom 14% were heteroplasmic. Patients with >25% wild-type mtDNA were not affected and there was a non-significant trend suggesting that heteroplasmic individuals were more likely to remain asymptomatic. Where
heteroplasmy did exist there was a drift towards homoplasmy in subsequent generations.

Overall it seems likely that heteroplasmy does play a role in disease expression, however it is not applicable to the majority of individuals. The exact contribution is further hampered by the unpredictable age at onset as many subjects classified as unaffected in the above studies may yet become symptomatic.

2.4.6.2 Genotype shift

Despite the genetic drift towards homoplasmy discussed above, the mutant load from a heteroplasmic mother may be differentially distributed amongst her children, with some receiving higher loads than others. In addition some patients classified as homoplasmic in fact have small amount of wild-type mtDNA undetectable by routine methods (Smith et al. 1993). This combination of factors makes the potential genotype of offspring is hard to predict with certainty.

2.4.6.3 Tissue distribution

In homoplasmic individuals LHON mutations are not surprisingly found in all tissue so far examined including muscle (Larsson et al. 1991), optic nerve (Howell et al. 1994), lymphocytes (Majander et al. 1991), platelets (Parker et al. 1989), and fibroblasts (Cornelissen et al. 1993). However in a heteroplasmic patient the mutant load may differ between tissues as is commonly seen in other mitochondrial disorders. It is clearly not practical to test the optic nerve in LHON and samples from elsewhere, usually blood, may not reflect the mutation load in the tissue of interest (Lott et al. 1990).

2.4.7 Genetic counselling in LHON

Given the variability in disease expression even where molecular confirmation is possible, genetic counselling in LHON families hampered by our
sparse knowledge of other influencing factors. MtDNA analysis is primarily useful to confirm diagnosis in suspected cases. We can reassure male carriers/patients that their offspring are not at risk, and families with the 14,484 mutation can be advised that the chance of useful recovery approaches 50% if the age at onset is under 20 years (Mackey, 1994). Information regarding the likelihood of clinical symptoms in the presence of a given mutation sought by concerned maternal relatives must be accompanied by explanation that such population derived figures cannot be directly applied to individuals. The study of 85 families by Harding et al. (1995) indicates recurrence risks of 30% (brothers), 8% (sisters), 46% (nephews), 10% (nieces), 31% (male matrilineal first cousins), and 6% (female matrilineal first cousins). In addition affected females in this study were more likely to have affected children than unaffected.

2.5 Mitochondrial morphology in LHON

Mitochondrial diseases are commonly characterized by abnormal mitochondrial morphology, predominantly studied in muscle specimens. Muscle is not clinically affected in typical LHON but none-the-less some have looked for structural changes in LHON mitochondria. The majority of reports predate genetic testing, but the cases are clinically typical with no symptomatic muscle involvement. Nikoskelainen et al. (1984) found sub-sarcolemmal aggregates of abnormally enlarged mitochondria in 8/13 patients and 1/9 controls. Such findings, though in a much more severe form are typical of mitochondrial myopathies and thought to represent an attempt to compensate for metabolic defects (Wallace, 1992). Other groups report similar findings (Uemura et al. 1987; Federico et al. 1988) but overall their numbers are small and controls not well age-matched. The incidence of such changes in larger control populations is unknown, and difficult to assess as healthy control muscle is hard to obtain for ethical reasons. Other changes characteristic of mitochondrial disease such as para-crystalline inclusions were lacking in all but 1 patient (Larsson et al. 1991).
The importance of these findings in LHON muscle is probably more that they contributed to the hypothesis that LHON might be a mitochondrial disease, rather than that they are of clinical significance themselves.

2.6 Mitochondrial Function in LHON

A crucial step towards understanding the pathogenesis of LHON is the study of mitochondrial function in subjects with associated mtDNA mutations. In other mitochondrial diseases pathogenetic mtDNA mutations are clearly associated with impaired MRC function, although the precise molecular mechanisms are poorly understood. Thus in LHON we might expect impaired complex I function given that the three primary mutations all affect complex I subunits. To date the data is conflicting and in limited numbers of patients.

2.6.1 Histochemistry

Staining muscle biopsies for specific mitochondrial enzymes though not quantitative gives a crude qualitative assessment of enzyme function. ATPase staining has always been reported as normal in LHON, and other stains for succinate dehydrogenase and the Gomori trichrome stain have shown only minor distribution abnormalities but no definite histochemical defect (Uemura et al. 1987; Federico et al. 1988), even though these same studies reported structural changes as discussed above. Again these represent very few patients and it might be argued that muscle is an irrelevant tissue in LHON. Both studies predate molecular diagnosis and so cannot be correlated to mtDNA mutations.

2.6.2 MRC enzyme activity in LHON

The Queensland family later shown to have both 14,484 and 4,160 mutations were the first in whom a clear biochemical defect was seen, with a 50-60% reduction in rotenone sensitive NADH:ubiquinone oxidoreductase activity (complex I) in platelet mitochondria from all 4 affected patients (Parker
et al. 1989). This supports a causative role for one of these mutations in MRC dysfunction, and for Complex I in disease pathogenesis. Biochemistry in isolated 14,484 LHON has not been reported.

Further work has consistently shown a similar severe complex I defect in 3,460 lymphocyte (Majander et al. 1991) and platelet (Smith et al. 1994) mitochondria. More specifically this mutation does not affect the proximal NADH dehydrogenase activity, only that of ubiquinone- dependent rotenone-sensitive electron transfer. This is in accordance with the proposed role of ND1 in rotenone and ubiquinone binding (Singer and Ramsay, 1994).

The situation in 11,778 LHON is less clear. Magnetic resonance spectroscopy (MRS) using P$^{31}$ as a non-invasive assessment of energy metabolism in vivo has shown alterations in muscle metabolism both during and after exercise in three 11,778 siblings, both affected and asymptomatic (Cortelli et al. 1991). The 11,778 subjects had higher metabolic activation than controls for comparable levels of relative work, and a reduced ability to recover phosphocreatine, taken to represent mitochondrial ability to synthesize ATP after depletion by exercise. Spectrophotometric enzyme activities on muscle mitochondria from the same individuals were normal, though rotenone sensitive NADH:ubiquinone reductase was not itself measured. The authors considered MRS to be more representative of the in vivo state, and concluded that spectrophotometric assays were insufficiently sensitive. Others have also reported normal electron transfer activity on spectrophotometric assays of rotenone sensitive NADH:cytochrome c oxidoreductase in muscle, (Larsson et al. 1991) and NADH:ubiquinone oxidoreductase in lymphocytes (Majander et al. 1991). However both groups also showed a reduced rate of oxygen consumption measured by polarography in the presence of complex I linked substrates, again suggesting a functional defect in intact mitochondria which is not detected by spectrophotometry on mitochondrial membrane fragments. Cigarette smoking is known to affect platelet complex I activity (Smith et al. 1993), and a mild (25%) defect in NADH:ubiquinone oxidoreductase activity.
was seen in the only study to exclude smokers (Smith et al. 1994). This factor may have masked mild defects in previous studies. Alternatively the defect may be a subtle one that is most evident in more physiological preparations. The most recent reports in this area show a reduction in rotenone sensitivity in 11,778 LHON, decreased affinity for a ubiquinone analogue with inhibitory activity (ubiquinone-2), and a less marked increase in the affinity of complex I for the substrate ubiquinone-1 compared to controls (Esposti et al. 1994). Such resistance to rotenone binding could account for a mild defect in complex I spectrophotometric assays, though this group found no such defect. The authors propose that such functional alterations in substrate binding might result in a partially reduced energetic efficiency of LHON complex I within an intact MRC, which might become critical in relation to other aetiological factors and result in disease. A brief follow up report (Carelli et al. 1995) claims to be able to distinguish affected from non-affected individuals on the basis of rotenone sensitivity which if true would be the first such marker. However numbers were very small and all groups overlapped, so further work is required in order to confirm or refute this.

Thus the functional consequences of LHON mtDNA mutations are not clearly understood, and although the findings to date in 3,460 LHON are consistent, they are at odds with studies on the more common 11,778 mutation. Further data on enzyme function and kinetics are needed in order to enhance our understanding of the molecular mechanisms involved in the pathogenesis of LHON.

2.7 X-linked / nuclear genes in LHON

Whatever the role of mtDNA mutations in LHON, the excess of male sufferers cannot be explained on the basis of mitochondrial genetics alone. An interaction with genes on the X-chromosome might be one explanation. Bu and Rotter (1991), using segregation analysis on 1200 individuals from 31 genetically confirmed LHON families, proposed that the disease penetrance
pattern is best explained by a 2-loci model involving both X-linked and mitochondrial genes. The occurrence of affected females could arise from homozygosity for the abnormal nuclear gene, or more commonly from X-inactivation within the embryonic cells that would later form the optic nerve. In contrast Mackey (1993) analysing data from 21 Australian pedigrees, noted that the observed frequency of blindness in the offspring of blind females with LHON did not fit with the frequency that an X-linked susceptibility gene would predict, if either dominant or recessive. However an X-linked susceptibility locus has also been supported by Harding et al. (1995) in a study of 85 LHON pedigrees. With this in mind a number of groups have attempted to associate LHON to an X-chromosome marker by linkage analysis. Initial claims of clear linkage to the locus DXS7 on the proximal short arm (Vilkki et al. 1991), have not been validated (Chen et al. 1989; Sweeney et al. 1992). Methodological differences make interpretation difficult, and in each study the penetrance was set lower than subsequent models have suggested. Large parts of the X-chromosome have not yet been excluded and it is also conceivable that different loci might be involved in different pedigrees. Thus this question remains unresolved.

2.8 Auto-immunity and LHON

The possible association between LHON and MS (Chapter 2.1.2), the micro-angiopathic appearance of the optic disc vessels in the acute stages and the presence of oligoclonal bands in the CSF (Pallini et al. 1988) have raised the possibility of auto-immune mechanisms in pathogenesis. Histocompatibility antigens have been linked to auto-immune diseases and several of the women with the 11,778 mutation and other neurological features compatible with MS reported by Harding et. al in 1992 had the HLA-DR2 antigen, strongly associated with MS in northern European populations. Following this lead the same group investigated the frequency of HLA-DR genotypes in 230 individuals from 79 families with known primary LHON mutations (Govan et al. 1994). No
association with any HLA-DR genotype could be found, and affected relative pairs did not share HLA genotypes more than discordant pairs. This suggests the HLA-DR locus is not a major genetic determinant for the development of blindness in LHON.

In rodents an immune response directed against a mitochondrial protein has been demonstrated. This maternally transmitted minor murine histocompatibility antigen is encoded by mtDNA (maternally transmitted factor; Loveland et al. 1990) and is presented on the cell surface by a class I molecule, as is usual for intracellularly derived peptide fragments. It is possible that similar mechanisms could operate in LHON and investigation of class I HLA antigens might be more revealing.

Another approach identified circulating antibodies to a tubulin protein in serum from 50 - 80% of affected LHON patients, 25 - 55% of unaffected relatives but only 14% of disease and healthy controls (Smith et al. 1995). The paucity of these antibodies in non-genetic optic neuropathies suggests they are not a non-specific response to optic nerve damage, but might be involved in disease pathogenesis. That some individuals carrying the antibodies remain unaffected is not an uncommon feature of auto-immune disease. Further work in this area is clearly indicated.

2.9 Questions remaining in LHON

Apart from the male excess, several clinical and biochemical features of LHON remain unexplained. The tissue specificity of the clinical defect is striking, despite the fact that associated mtDNA mutations have been documented in all the tissues so far studied. It has been proposed that this might reflect higher mutation loads in the optic nerve than elsewhere (Howell et al. 1994), but the presence of 100% mutation in a given tissue is not sufficient to cause a clinical defect as has been regularly found in the healthy blood, muscle or skin of LHON patients. Other factors must be involved. The high oxidative demands of brain and muscle tissue is often cited as a reason for the
frequent involvement of these organs in other mitochondrial diseases, but it is
difficult to apply this reasoning to LHON.

The possible role of X-linked genes in LHON has already been
discussed, but even this bigenomic model may provide insufficient explanation.
Monozygotic twins with the 11,778 mutation and implicit identical nuclear
backgrounds have been described of whom only one is affected with onset at
34 years (Johns et al. 1994). The unaffected brother, at 41 years may yet
become symptomatic, but the least that this report implies is non-genetic
influences on the age of onset, if not on disease penetrance itself. The potential
role of environmental hazards such as smoking, alcohol and other metabolic
upset in precipitating or contributing to disease remain open to debate.

Finally the functional consequences of primary mtDNA mutations are not
clearly defined, and no work on complex I structure in such patients has been
published. Clarification of these issues may help in understanding the
molecular mechanisms underlying the clinical picture. We might also study the
influence of the nuclear environment in *in vitro* systems. Such information is
essential if we are to eventually provide better guidance for individual carriers,
and perhaps offer hope of prevention or treatment.
Chapter 3. Ageing and Hutchinson-Gilford progeria syndrome (HGPS)

Ageing is an inevitable accompaniment to life, and is a process that many would seek to retard if not reverse. Not surprisingly perhaps there is an enormous wealth of literature directed towards understanding the underlying processes. That mitochondria might be involved in the ageing process, particularly through the generation of free radicals is not a new idea (Harman, 1969), but one that has received much attention. Before covering this field in some detail I will first briefly review the clinical features of normal ageing and other broad theories. I will then discuss a disease often cited as a model of accelerated ageing - Hutchinson-Gilford progeria syndrome (HGPS), and the aims of this work in studying mitochondria in HGPS.

3.1. Features of normal ageing

Ageing has been defined as the progressive accumulation of changes with time that are associated with, or responsible for, the ever increasing susceptibility to disease and death which accompany advancing age (Harman, 1981). Beyond this clinical definitions are fraught with difficulty - when does a disease process which is commoner in the elderly, e.g. atherosclerotic disease, become a feature of normal ageing? Other time dependent changes, e.g. embryo-genesis, deciduous teeth and puberty are considered as developmental and not to represent senescence, but where are such distinctions drawn? Ageing involves a progressive loss of adaptation and function together with a decreasing expectancy of life, both at the clinical and molecular level with no compensatory new structures of functions. It is this that distinguishes it from other time dependant processes.

Commonly accepted clinical features of normal ageing include loss of or greying of hair, wrinkling of the skin, impairment of sight and hearing, atherosclerosis, and a minor decline in cognitive function (Goldstein, 1971; Morris and McManus, 1991). Diseases such as diabetes mellitus, cancers, and most of the commoner neurodegenerative conditions (Alzheimer's disease,
Parkinson's disease and motor neurone disease) whilst not a definite part of "normal ageing" are much more common with advancing age and as such may share pathogenetic mechanisms. An already declining cell population due to ageing might lower the threshold at which such clinical phenotypes emerge.

Attempts to find quantifiable biological measures of ageing such as a fall in renal blood flow, in cardiac output or bone density (Rowe and Kahn, 1987), or changes in cerebral metabolism and blood flow (Marchal et al. 1994), whilst demonstrating a downward trend reveal considerable variability between individuals. Thus the aged phenotype has some consistent features but is enormously protean in clinical manifestations. It is no wonder then that there are many "sub-theories" which have evolved to account for the changes seen. Most of them probably explain some aspects of a complex multi-factorial process.

3.2 Non-mitochondrial theories of ageing.

3.2.1 Constitutional genetics of ageing

Genetic markers of ageing can be considered in two categories. First constitutional genes which might determine the rate/initiation of one or more of the processes of ageing, and secondly acquired somatic damage to DNA which could result in a loss of cellular functions.

For reason's outlined above measurement of ageing is complex but that one's own genetic makeup influences longevity is in little doubt. Each species has a characteristic mean and maximum life-span, and in man there is a positive correlation between the life-span of parents and their offspring (Goldstein, 1971). Twin studies also support a role for nuclear genes in determining life-span (Martin, 1994). Identification of genes that could be involved is complicated by the diverse changes see in ageing, though some specific associations with extreme old age have been reported: polymorphisms in the genes encoding apolipoprotein E, a major protein in lipid metabolism, and that for angiotensin converting enzyme important in salt and water homeostasis.
and control of cell growth, have been found in high frequency in French centogenerians (van Bockxmeer, 1994). Other alleles in the same genes are implicated in the pathogenesis of cardiovascular and neurodegenerative disease, but the mechanisms contributing to longevity remain indeterminate.

3.2.2 The somatic mutation theory

First put forward by Szilard in 1959 this theory proposes that the genome is subject to a constant barrage of "ageing hits" throughout life, progressively damaging and inactivating genes in a random fashion. At a critical threshold of damage the probability of an individual dying would approach 1. Along the pathway to this the phenotypic features of ageing would accumulate in a random manner in any given individual. Consistent with this theory is the increase in chromosomal breaks and aneuploidy with advancing age (Goldstein, 1971), but though causes of death and disease accumulate with time they bear no determinate relation to the number of mutations in the body (Comfort, 1969). In addition this theory would predict that the life-span of a species is proportional to the DNA content of its genome which has not been borne out. Hence random somatic mutation cannot be a sole cause of ageing and death, and such a theory takes no account of known non-genetic disease processes.

3.2.3 Telomeres and telomerase in ageing

One aspect of ageing which is much studied is that of replicative senescence i.e. the loss of proliferative ability in normal (un-transformed) somatic cells (Goldstein, 1990). Human fibroblasts for instance have a finite replicative capacity in vitro, which shows little correlation with the chronological age of the donor. Much evidence now exists suggesting that the cause of this cellular senescence is the gradual loss of telomeres (Harley, 1991). Telomeres play a critical role in chromosome structure and function, preventing aberrant recombination and attaching the chromosome ends to the nuclear envelope.
(Greider, 1990). They are composed of DNA repeats at the 3' end of the chromosome, and are maintained by a particular DNA polymerase called telomerase. Chromosomes lacking telomeres are very unstable and could contribute to dramatic changes in cell function. It has been shown that telomeres shorten during *in vitro* ageing of many cultured somatic cells, and that this is accompanied by increasing chromosomal aberrations (Harley, 1991). Immortal cell lines have levels of telomerase activity which seems to balance telomere loss, and more recently it has been shown that initial telomere length predicts replicative capacity of human fibroblasts in culture (Allsopp *et al.* 1992). The telomere theory of cellular ageing then hypothesizes a progressive loss of telomeres due to incomplete DNA replication and absence of telomerase in somatic cells providing a mitotic clock that ultimately signals cell cycle exit. Only a weak correlation exist between donor age and telomere length but this might represent very different starting points for individuals on which a constant rate of telomere loss is superimposed (Martin *et al.* 1993). Monozygotic twins show greater concordance for telomere length than dizygotic twins, regardless of environment, so genetic factors presumably influence telomere length (Martin, 1994). Further work on the mechanisms by which telomere loss might contribute to the *in vivo* situation is likely to emerge over the next few years, though the limitations of the somatic mutation theory will inevitably apply here also.

### 3.2.4 The error catastrophe theory of ageing

This postulates that increasing errors in transcription and translation lead to a protein synthetic machinery with progressively lower fidelity, and to the eventual accumulation of a lethal proportion of aberrant proteins in the cell (Goldstein, 1971). Thus cells from older donors would be expected to show higher error frequencies. Senescent cells in culture would also show high error rates, whereas immortal/transformed cells might be expected to have low rates if protein synthetic errors are a major determinant of cell life-span. Studies of
mis-translation on human cells however have not supported this theory as cells from older donors, or senescent cells in culture did not show significantly elevated rates of mis-translation compared to fetal/young cells (Harley et al. 1980).

3.2.5 Apoptosis and ageing

Apoptosis has long been recognized as having a wide incidence throughout the animal kingdom particularly during development and reproduction. The definition encompasses an orderly and characteristic sequence of structural changes seen during physiological cell death, defining built-in cellular mechanisms which can be triggered by extra-cellular factors (Wyllie et al. 1980). The first stage is nuclear and cytoplasmic condensation and breaking up of the cell into a number of membrane bound, ultra-structurally well preserved fragments. These apoptotic bodies are then shed from epithelial lined surfaces or taken up by other cells where they are rapidly degraded by lysosomal enzymes. Apoptosis as an active inherently programmed phenomenon has a clear role for instance in embryo-genesis: neurones dying during synapto-genesis, lymphocytes dying during receptor repertoire selection, degeneration of the Müllerian duct in male mammals. The notion that in adult organisms such a “suicide program” might exist is more recent (e.g. elimination of lymphocytes possessing self-reactive receptors, elimination of potential cancer cells) - reviewed by Raff (1992). Evidence also exists for apoptotic mechanisms in neurological diseases (Bredesen, 1996). If this process is so widespread might it not also contribute to tissue decline in ageing? There is evidence that the body's cell mass significantly declines with age, particularly in several areas of the brain (Morris and McManus, 1991), but as yet reports of a role for apoptosis in ageing remain appealing but speculative (Goya, 1986).
3.3 Free radicals/ oxidative damage and ageing

Free radicals are molecules with one or more unpaired electrons that are generally unstable. They are ubiquitous in living systems and produced during normal cellular metabolism. Oxygen species in particular, e.g. super-oxide ($O_2^-$) and hydroxyl ($OH^-$), are postulated to result in a multiplicity of deleterious changes in biological systems through random oxidative reactions with cell constituents. These include genomic DNA, lipids, proteins and mitochondrial structures. Not surprisingly the body has major defence systems against oxidative stress including potent antioxidants such as ascorbic acid, glutathione peroxidase, catalase, super-oxide dismutase and vitamin E, as well as mechanisms to detect and repair/replace damaged molecules (reviewed by Martin et al., 1993). In the normal state a balance of oxidative stress, antioxidant defences and host cell repair mechanisms presumably exists. The free radical theory of ageing postulates disruption of this balance such that free radical damaged structures persist and/or defences are overcome, eventually resulting in a decline in cellular function and death. First proposed in 1956 (Harman) much work has followed in support of and expanding on this hypothesis. There is now a growing body of evidence free radical damage to cellular function is associated with many age-related disease e.g. atherosclerosis, arthritis, cataracto-genesis, neurodegeneration and cancers. To cover each of these in any detail would not be relevant, so I will concentrate on that work directly related to increasing age.

Mitochondria and the respiratory chain enzymes are recognized as the most potent source of free radicals in the cell (Cadenas et al. 1977), so any discussion of free radicals in ageing is unavoidably linked to theories involving mitochondrial dysfunction and damage. Any or all of the changes identified in ageing mitochondria might arise through a free radical mediated model. Mitochondria will be discussed after considering the evidence that oxidative damage to other cellular structures might contribute to the ageing process (reviewed by Pacifici and Devies, 1991).
3.3.1 Oxidative damage to genomic DNA

Most studies looking for evidence of oxidative damage rely on detecting increasing amounts of oxidatively modified molecules with increasing age. In the case of nuclear DNA the commonest technique has been to measure the level of modified purine or pyrimidine bases (e.g. 8-hydroxy-deoxyguanidine: OH8dG). Several studies have confirmed an increase in nuclear OH8dG with age in animals (Adelman et al. 1988) and in man (Mecocci et al. 1993). As free radicals are produced by normal metabolism we might expect that species with a high basal metabolic rate (BMR) are more susceptible to oxidative damage, and if the ageing theory is true have shorter life-spans. Data from mice, rats, monkeys and man show that the BMR, oxidative DNA damage and life-span are all positively correlated - suggesting that genomic DNA is a critical target in ageing (reviewed by Ames, 1989). Against this nuclear DNA is compartmentalized away from mitochondria and peroxisomes where free radicals are largely generated, most non-replicating nuclear DNA is surrounded by protective histones and polyamines and finally, most DNA damage can be repaired by efficient enzyme systems. Presumably the net result of this multi-level defence is that nuclear DNA is well, but not completely, protected.

3.3.2 Protein oxidative damage

That direct oxidative damage to proteins occurs is not a subject in dispute. Despite this it's potential role in ageing was disregarded until recently. This may reflect the lack of evidence supporting the error catastrophe theory turning scientific attention away from proteins, and that it seemed reasonable to assume that damaged proteins would not accumulate greatly given the high turnover rate of cellular proteins, particularly if damaged. However there are now several experimental systems to detect oxidatively modified proteins, and support is now widespread for accumulation of oxidatively damaged proteins with age in a variety of models including in human ageing (reviewed by Stadtman, 1992). This is likely to be the result of a combination of increased
production of oxygen free radical mediated damage and a loss in the ability to
degrade oxidized proteins. As proteins are involved in an enormous range of
cellular enzymatic functions and fundamental constituents of supportive tissues
(e.g. collagen) such damage could have far reaching and diverse effects.

3.3.3 Lipid peroxidation

Lipids are a fundamental constituent of all cells and cell membranes. Oxidative damage to lipids can be measured in a variety of ways, mostly concentrating directly on the presence of oxidated lipid metabolites. In numerous animal studies increased lipid peroxidation has been shown to occur in ageing brains (Mooradian and Uko-eninn, 1995), heart and liver (Sawada et al. 1992). Plasma malondialdehyde (the most commonly measured marker) also increases in man with age (Rodriguez-Martinez and Ruiz-Torres, 1992), and there is an age dependant accumulation of lipids that are more prone to peroxidation (Shigenaga et al. 1994). The significance of such damage lies in its functional effects. These include a change in membrane fluidity, membrane leakage, and secondary effects on the many protein/enzyme systems embedded within or dependant on a protected membrane bound environment.

3.4 Mitochondria and ageing

The possible role of mitochondria in ageing is by now clear given the link to free radicals and oxidative damage to other cell structures as discussed above. One proposal is that the primary abnormality is within the mitochondria itself (Shigenaga et al. 1994), leading into a vicious cycle whereby a dysfunctional MRC leads to enhanced free radical production, overwhelming host defences and causing a further decline in respiratory capacity. Some take this further and have proposed that the central site of irreversible injury is the mtDNA itself rather than the biomembranes of the cell (Fleming et al. 1982). This is an attractive hypothesis for several reasons. Firstly mtDNA is situated close to a major site of free radical production and lacks the protective and
repair mechanisms of genomic DNA. Secondly significant damage to mtDNA would be expected to impair MRC function, which in turn leads to further free radical production and potential oxidative damage both to the mtDNA and to the MRC proteins (Zhang et al. 1990). Finally cellular ATP is fundamental to many physiological functions. Thus a vicious cycle would be established leading to a fall in the production of cellular ATP, loss of energy dependant cellular functions and ultimately cell death. In this section I shall review in some detail the evidence for accumulated mtDNA damage and a fall in MRC function with ageing.

3.4.1 Mitochondrial DNA and ageing

3.4.1.1 Oxidative damage to mtDNA and the "common" deletion

In accordance with the mitochondrial theory of ageing there is evidence that mtDNA is more susceptible to oxidative damage with increasing age than nuclear DNA. Using similar study methods to those on nuclear DNA, the increase of OH8dG in mtDNA with advancing age appears considerable, with a 70 year old human brain displaying a 15-fold excess over that found in infants (Mecocci et al. 1993). The same is true in post-mortem heart samples (Hayakawa et al. 1992) and the increase exponential as might be predicted. This is merely evidence of oxidation to mtDNA bases, but there is no doubt that the prevalence of identifiable mtDNA mutations also increases with age. Large scale sequence changes such as deletions have been easiest to detect, initially using southern blotting and more recently using PCR based methods for particular known sequence changes such as the "common" 4,977bp deletion associated with KSS and Pearson's syndrome. Applied to various post mortem tissues in subjects ranging from fetal/neonatal to over 90 years old, numerous studies have demonstrated that the common deletion starts to be easily detectable in most adult human tissue during middle age and increases both in prevalence and quantity with advancing years e.g. (Linnane et al. 1990; Cortopassi and Arnheim, 1990). A similar increase in deleted mtDNA with age
has been confirmed in muscle and skin samples from living donors without known mitochondrial disease (Hsieh et al. 1994; Yang et al. 1994).

In keeping with the mitochondrial hypothesis those tissues most susceptible to oxidative stress, namely brain and muscle, have more common deletion than others with lower metabolic rates such as liver, kidney or spleen (Cortopassi et al. 1992). Combined information from these studies suggests that in brain and muscle by 30 years of age 50% of us have sufficient mtDNA with the common deletion to be detectable by 30 PCR cycles and by 50 years this is nearly 100%. In skin and other tissues prevalence is lower, first appearing in the 60's in 20% of subjects, 50% in the 70's and found in over 80% of us who reach 80 years.

Absolute quantification of levels is difficult, and no method infallible. Most studies that attempt this can at best only offer estimates. Scintillation counting of radioactively labelled PCR products, compared to those of a standard curve constructed from known proportions of deleted/complete mtDNA proposes a maximum of 0.1% deleted mtDNA or 1/1000 molecules in the >70 year old age group (Cooper et al. 1992b), approximating to an average of 1 deleted mtDNA molecule per cell. This is where the mitochondrial theory starts to have problems. Even allowing for the fact that some deletions may cluster together in a single cell, it is hard to imagine such a low level could really contribute significantly to functional decline.

3.4.1.2 Other mtDNA mutations

One explanation for this problem is that the common deletion represents only the "tip of the iceberg" being only one manifestation of much more extensive mtDNA damage. There are fewer studies addressing this but those that do are in support. Increasing levels of tandem duplications (Lee et al. 1994), other deletions (Baumer et al. 1994) and a 3243 point mutation (Zhang et al. 1993) have all been demonstrated in human tissues with increasing donor age. Individually each still only represents a tiny amount of total mtDNA, but
together with possibly thousands of different as yet undetected mutations the total mutant load might be considerable, and could be capable of affecting MRC function.

3.4.1.3 Other work on mtDNA and ageing

One important paper by Hayashi et al. (1994a) concludes that whilst a progressive biochemical defect in MRC function was seen with increasing donor age, as will be discussed in the next section, nuclear and not mitochondrial genes are responsible for the functional decline. Fusing enucleated fibroblasts from elderly donors with a transformed mtDNA-less cell (ρ0HeLa cells), thus placing "elderly "mitochondria in a new nuclear environment, resulted in restoration of mitochondrial function to levels comparable to those of younger subjects. If validated in other laboratories this would effectively rule out a significant role for mtDNA mutations in ageing.

A second study against aspects of the mitochondrial/ oxidative damage theory of ageing has been performed by Moraes et al. (1995). This took the basic principle that if oxidative damage plays a role in generating mtDNA damage in ageing, specifically the common deletion, then patients with mitochondrial diseases and consequent high levels of oxidative stress should also display higher levels of deleted mtDNA than would be expected for their age group. Quantitative PCR on muscle samples from healthy subjects confirmed an increase in the amount of deleted mtDNA with age, though the confidence limits were considerable with up to a thousand fold variation at any given age. Similar analysis on samples from patients with MERRF/ MELAS and CPEO did not show any excess of the common deletion above that expected for the patients age-group. This implies that local oxidative stress is not a major factor influencing the increasing common deletion seen with age, though the sensitivity of the technique may be inadequate to draw firm conclusions.
3.4.2 MRC function and ageing

3.4.2.1 Complex I

Studies on mitochondria prepared from human muscle have shown a significant decline in NADH:ubiquinone oxidoreductase specific activity with age such that subjects in the 70 - 90 year age group had only 50% the activity of subjects in their 20's.\((r = -0.667, p = 0.032;\) Cooper et al. 1992b\). The same trend was seen in polarographic studies with NAD-linked substrates \((r = -0.767, p = 0.016)\). Similar findings have been reported by others in human muscle (Hsieh et al. 1994; Blin et al. 1994), and in ageing monkey brain mitochondria (fronto-parietal cortex; Bowling et al. 1993). Consistent with these findings assays of ATP production, again from monkey brain mitochondria (striatum), have also showed a negative correlation between NAD-linked ATP production and advancing age (Di Monte et al. 1993).

Others have failed to validate this data but either the numbers have been insufficient (Cardellach et al. 1993 - only 8 subjects) or the tissue studied not appropriate. The normal life-span of platelets in the circulation for instance is only 7 - 10 days, so they might not be expected to show chronic accumulative damage such as proposed for ageing (Bravi et al. 1992).

Significant changes in complex II or III activity with age are not commonly reported.

3.4.2.2 Cytochrome oxidase/complex IV

Several groups have reported a correlation between the numbers of COX negative ragged red fibres and age in ante-mortem and post-mortem muscle samples. This is true in cardiac (Müller-Höcker, 1989), diaphragmatic and skeletal limb muscles (Muller-Höcker, 1990; Rifai et al. 1995). In keeping with this a fall in COX activity with increasing donor age has been demonstrated on mitochondria prepared from human muscle (Cooper et al. 1992b; Hsieh et al. 1994), platelets (Van Zuylen et al. 1992), cultured fibroblasts (Hayashi et al. 1994a) and brain (Kish et al. 1992). Given the overall consistency of this data a
decline in COX activity may have a critical role in the ageing process. Groups who did not observe any significant decline with age, as for complex I data, had smaller numbers which may account for this (e.g. Solmi et al. 1994).

3.4.3 Conclusions

There is considerable data then showing evidence of oxidative damage to many cell components with advancing age. I have not considered the data on antioxidant defences in relation to this. Although a huge amount of data exists, the results are neither consistent or helpful. Either increasing or decreasing antioxidant defences with age either can be interpreted as in favour of ongoing oxidative stress. An increase might represent a positive feedback system, whereas a fall might be considered indicative of a defence strategy overwhelmed by powerful attack. Moreover the putative enzymes involved are increasing in number such that any comprehensive review would be considerable in length, and not directly relevant to this work.

That mitochondria are involved somehow both in free radical production and in ageing seems well supported. The problem lies in identifying which, if any, of these changes depict primary pathogenetic mechanisms, and which are merely secondary markers of a more generalized functional decline. This question remains unsolved.

3.5 Using models of ageing for research

From the information so far it will be apparent that the aged phenotype is most likely the end result of many cascades of events stemming from several distant sources. The combined effects may be too complex to clearly separate out in order to make a realistic assessment. In addition human ageing by nature takes years to evolve. One approach to these problems is to limit study to particular aspects of the ageing process or to look for disease models of accelerated ageing. With this in mind several syndromes which share some of the clinical features of normal ageing have been cited as models of accelerated
ageing. Most of these are rare and by definition none are truly representative. However study of such patients may allow aspects of the ageing process to be unravelled.

3.6 The Hutchinson-Gilford progeria syndrome (HGPS)

In this section I will concentrate on one particular condition, Hutchinson-Gilford progeria syndrome (HGPS), as this is generally considered the best model and we have had the opportunity to study two affected patients. Other conditions also quoted as examples of accelerated ageing or listed in Table 2.

3.6.1 Clinical features of HGPS

Progeria is a term sometimes applied to several diseases in which the individual appears prematurely aged. HGPS is a much more clearly defined condition with a very distinct clinical phenotype. First outlined in 1886 and the early years of this century (Hutchinson, 1886; Gilford, 1904) some 75 cases have since been described in the literature (60 of whom are reviewed by DeBusk, 1972). This over-represents its true incidence which is in fact extremely low. The United States reports only 1 case per 8 million births (DeBusk, 1972). The very consistent clinical phenotype suggests a genetic causation. There have been reports of affected siblings and cousins from unaffected consanguineous parents (DeBusk, 1972; Khalifa, 1989) suggesting autosomal recessive inheritance, but with such small numbers inheritance patterns are difficult to substantiate.

The clinical features of HGPS as reviewed by several authors are summarized here (DeBusk, 1972; Mills and Weiss, 1990; Brown, 1992). At birth most appear normal but present within the first year of life with failure to thrive or skin/hair abnormalities. Both height and weight deficits become rapidly apparent, the latter most marked with an invariable striking lack of subcutaneous fat. There are consistent cranio-facial abnormalities giving a "plucked-bird" appearance with micrognathia, mid-facial cyanosis, a
"sculptured" nose, prominent scalp veins and alopecia. Dentition is abnormal and delayed, and complete sexual maturation does not occur. Skeletal abnormalities such as short dystrophic clavicles, a pyriform thorax and coxa valga, are common. The skin typically appears "aged" being thin and dry with little hair, prominent veins and many loose wrinkled areas even at five years old. Other regions may seem scleroderma like with tethering and a smooth shiny appearance. Later irregular pigmented areas appear and there may be dystrophic nail changes. Arthritis and thinning bones are common but differ radiologically and pathologically from the osteoarthritis and osteoporosis accompanying old age. Intellect and cognitive function are normal, though may be later affected by cerebrovascular disease. Vascular complications are the commonest cause of death as there is widespread premature atherosclerosis. Cardiac vessels are most severely affected often causing angina by the age of 10, and myocardial infarctions/cardiac failure in the teens. The mean age at death is 13.4 years with a range of 7 to 27 years.

No demonstrable abnormalities of endocrine function have been found other than insulin resistance (Villee et al. 1969), which may also be a feature of normal ageing. None of the reported HGPS cases have demonstrated other aspects of old age such as senile cataracts, presbyacusis, arcus senilis, osteoarthritis or the development of senile plaques/neurofibrillary tangles in the CNS.
<table>
<thead>
<tr>
<th>Disease/ syndrome</th>
<th>&quot;Ageing&quot; clinical features</th>
<th>Other major clinical features</th>
<th>&quot;lacking&quot; features</th>
</tr>
</thead>
<tbody>
<tr>
<td>HGPS</td>
<td>alopecia; skin changes; muscle and CT atrophy; atherosclerosis; arthritis; osteoporosis;</td>
<td>dysmorphic facies; abnormal dentition; congenital</td>
<td>cataracts; CNS changes; neoplasms</td>
</tr>
<tr>
<td></td>
<td>insulin resistance</td>
<td>skeletal abnormalities</td>
<td></td>
</tr>
<tr>
<td>Werner's syndrome</td>
<td>hair greying; cataracts; skin changes; muscle and CT atrophy; atherosclerosis; osteoporosis; neoplasms;</td>
<td>growth retardation; hypogonadism; diabetes; skin</td>
<td>CNS changes; prostatic hypertrophy; hypertension</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ulceration; soft tissue calcification</td>
<td></td>
</tr>
<tr>
<td>Cockayne's syndrome</td>
<td>skin, muscle and connective tissue changes; cataracts; deafness</td>
<td>growth retardation; mental deficiency; photosensitivity; hypogonadism; Neoplasms; CNS changes; CVS disease</td>
<td></td>
</tr>
<tr>
<td>Down's syndrome</td>
<td>dementia; neoplasms; cataracts</td>
<td>mental and growth retardation; hypogonadism; congenital vascular malformations</td>
<td>ageing skin/muscle changes; acquired CVS disease</td>
</tr>
<tr>
<td>Ataxia telangiectasia</td>
<td>neoplasms; increased spontaneous chromosomal abnormalities</td>
<td>congenital vascular abnormalities; impaired immune function; cerebellar ataxia; Ageing skin/muscle/CT changes; CNS changes; Acquired CVS disease</td>
<td></td>
</tr>
</tbody>
</table>

Disorders cited as models of accelerated ageing in man, illustrating the merits and limitations of using such models in the study of normal ageing. HGPS = Hutchinson-Gilford progeria syndrome; CT = connective tissue; CVS = cardiovascular system; CNS = central nervous system. References: (Goldstein, 1971; Mills and Weiss, 1990; Martin, 1988).
3.6.2 HGPS as a model of accelerated ageing

Assessment of a disease as a model of normal ageing requires that we distinguish features of "normal" ageing from disease processes, which as I have previously discussed is not easy. None of the proposed models in Table 2 express a full cross-section of the ageing phenomenon. Those who have considered the candidates in detail conclude that HGPS is the best model we have, though recognising it's limitations (Mills and Weiss, 1990; Brown, 1992). HGPS perhaps has more clinical homology than the others, and superficially has a more reliable onset and progression than for instance Werner's syndrome or ataxic telangiectasia. Moreover it has been observed that HGPS is manifested primarily in cells of mesodermal origin (Brown et al. 1985), providing a potential means whereby a single gene defect might exert a profound phenotypic ageing effect. Whilst no-one would suggests that HGPS truly represents an accelerated version of normal ageing, the study of such patients might reveal pointers to processes which both have in common and aid our understanding not only of this rare condition, but also of normal ageing.

3.7 Aetiological theories in HGPS

3.7.1 Studying HGPS

Two major problems face anyone wishing to study the cellular mechanisms in HGPS. Firstly it's rarity limits the availability of clinical material, and no animal models are known. Secondly most researchers find that as well as a reduced life-span in vivo, attempts to culture progeria cells in vitro are hampered by a limited replicative capacity. Control fibroblasts are expected to undergo around 40 - 50 population doublings (PDL) before showing signs of cellular senescence, but in cultures established from HGPS patients cessation of proliferation has been reported at only PDL 10 - 14 (Colige et al. 1994). Others have cultured fibroblasts for longer, but in all cases some degree of
limitation is seen with reduced growth rates, DNA synthesis and mitotic figures (Danes, 1971). The positive aspect of this is that premature senescence is clearly seen at a cellular level in HGPS fibroblasts as well as in the patient as a whole. Thus in vitro work might be able to identify at least some of the molecular mechanisms responsible.

3.7.2 Telomeres in HGPS

Telomere length has been proposed as a biomarker of somatic cell ageing as I previously discussed. If HGPS and ageing share cellular mechanisms then cells from HGPS donors, with their reduced division potential, might have shorter telomeres than would be expected on the basis of the donor age. The one study to directly assess this indeed found significantly shorter telomeres in HGPS donors (number not stated) compared to age matched controls (Allsopp et al. 1992).

3.7.3 DNA repair in HGPS

A major aetiological hypothesis proposes that a primary defect in HGPS is reduced capacity to repair damaged DNA. There is support for this: Epstein et al. in 1973 demonstrated reduced single strand repair after γ- irradiation of cultured HGPS fibroblast monolayers compared to controls, and most subsequent studies have consistently in demonstrated abnormal DNA repair as assessed by measuring unscheduled DNA synthesis or host cell reactivation in response to UV or γ- irradiation induced damage (Wang et al. 1990; 1991). This hypothesis is attractive as such a defect could ultimately result in widespread changes such as is seen in HGPS, though a specific abnormality in a widely distributed tissue component, such as collagen, could also be responsible.
3.7.4 Connective tissue proteins

As has already been pointed out those tissues predominantly affected in HGPS are of mesodermal origin, and clinically a large proportion of problems could be attributed to abnormalities of connective tissues and extra-cellular matrix proteins. One such protein, hyaluronic acid (HA), is implicated in functions such as vascularity, morphogenesis, repair and the general integrity of the extra-cellular matrix. Hence HA might be involved in the most conspicuous features of HGPS. A number of studies have shown increased excretion of HA in the urine of HGPS patients, suggesting a loss from the tissues and this is comprehensively reviewed by Sweeney and Weiss (1992). Increased HA excretion has also been seen in normal ageing, though this has been less well defined. Whether this represents a primary defect in HGPS or is merely a secondary marker of another process, for example a more generalized abnormality in proteolysis, remains to be clarified.

Along similar lines of thought others have studied additional important matrix protein systems. Beavan et al. (1993) found reduced protein and mRNA levels for decorin, an important matrix proteoglycan, in 5 patients with progeroid syndromes compared to controls, though only one satisfies the clinical criteria for HGPS. One particular glycoprotein, gp200, has been identified in HGPS fibroblasts and those of a 92 year old control, which is absent or much reduced in younger controls (Clark and Weiss, 1993). A follow-up study proposes a perturbation in glycosylation underlying the connective tissue defects seen in progeria (Clark and Weiss, 1995). Giro and Davidson (1993) found high levels of elastin production by HGPS fibroblasts and of note this was also seen in the mothers of all patients compared to controls, suggesting a hereditary component to connective tissue abnormalities.

Thus it seems there is general agreement that a defect in HGPS connective tissues exists, but it is not yet reliably defined, and none of the
reported changes may be of primary pathogenic significance on the basis of current evidence.

3.7.5 Oxidative metabolism in HGPS

Little work has looked at energy metabolism in HGPS. A detailed study of many aspects of metabolism in two patients by Villee et al. in 1969 reported normal polarography in muscle mitochondria with complex I linked substrates; other substrates were not examined. Again predating the interest in mitochondrial function and ageing, Goldstein et al. (1982) reported normal oxygen consumption (polarography), pyruvate and glutamate oxidation and ATP synthesis in fibroblasts from a single patient with HGPS compared to a single age matched control. However growth spurts in progeria fibroblasts were accompanied by an increase in lactate production suggesting there might be an oxidative metabolism defect not formally identified by the methods used. This has not been pursued and despite the wealth of work in ageing detailed mitochondrial function has not been reported in HGPS.

3.8 Aims of studying mitochondria in HGPS and ageing.

Given the existing evidence for mitochondrial dysfunction, and possibly for mtDNA damage, in ageing it seems logical to look at mitochondria in HGPS. It is not feasible to expect to understand all the mechanisms responsible for either ageing or progeria, but detailed study of this particular aspect might clarify the proposed role of HGPS as a model of accelerated ageing in this context, and also allow us to contribute to existing data on the MRC and ageing.
Part 2: Methods

Chapter 4. Materials and methods

4.1 Clinical details and cell lines

4.1.1 3460 LHON patients

Primary fibroblast cultures were established from 3 members of a family known to show heteroplasmy for the 3,460bp LHON mutation. The family tree is detailed in Appendix A. Ages given are as at the time of the skin biopsy.

IV₁  Affected male proband d.o.b. 1970, age at biopsy 24 years.
     Sequential central visual loss age 19 (left eye) and 20 (right eye) years. Visual acuity bilaterally 6/60. No clinical recovery. No other significant medical history or medication. Non-smoker.

IV₂  Unaffected brother of proband d.o.b. 1972, age 22 years.

III₂  Unaffected mother of proband d.o.b. 1946, age 47 years.
     Neither had any other significant medical history, were smokers or taking any medication.

4.1.2 11778 LHON patients

Lymphoblasts were purchased from the European Collection of Animal Cell Cultures, Porton Down, UK. The family tree is detailed in Appendix B.

IV₃  Affected male proband d.o.b. 1952, catalogue no. DDO138

IV₁₀ Unaffected male cousin of proband d.o.b. 1954, catalogue no. DDO311

IV₁₁ Unaffected female cousin of proband d.o.b. 1953, catalogue no. DDO312.
     No other details were available
4.1.3 14484 LHON patients

The proband (III₈) was known to us and family members were subsequently contacted. The family tree is detailed in Appendix C. Primary fibroblasts cultures were established from 3 members. Ages given are as at the time of the skin biopsy.

III₈  Affected male (proband) d.o.b. 1949, age 45 years.
Sequential painless loss of vision in both eyes in 1979. No recovery. Acuity hand movements only bilaterally. Also a heavy drinker (> 50 units/week) and smoker (40 cigarettes/day).
Past medical history: chronic obstructive airways disease; alcohol withdrawal fits in 1991 and 1993; pneumonia needing ventilation 1993; alcoholic peripheral neuropathy diagnosed 1993; psoriasis. Magnetic resonance imaging in 1993 showed mild cerebellar and cortical atrophy consistent with chronic alcohol excess; nerve conduction studies showed a mixed sensorimotor axonal polyneuropathy. All blood tests, cerebro-spinal fluid and CT were normal. Heavy alcohol and cigarette intake persisted at the time of biopsy. This subject died from the complications of alcoholic liver disease in April 1996. Further post-mortem details are awaited.

II₅  Unaffected female (mother) d.o.b. 1919, age 75 years.

III₃  Unaffected female (sister) d.o.b. 1955, age 39 years.
Neither had any significant medical history. Both were non-smokers and were on no medication.
4.1.4 HGPS patients

4.1.4.1 DP

DP was referred for investigation by his paediatrician, Dr. J. Harper (Northampton General Hospital), and primary fibroblasts cultures established from a skin biopsy taken in 1993 when he was 20 years old. DP presented at the age of 2 with failure to thrive. At that time his weight was below the 3rd percentile, but his head circumference close to the 50th percentile. There was a striking lack of hair, prominent scalp veins, a lack of subcutaneous fat and hypoplastic nipples. X-rays revealed abnormal skull bones and decreased vertical height of the vertebrae. His mental development was comparable to his chronological age. A diagnosis of Hutchinson-Gilford progeria syndrome was made based on the clinical features. He remained well below the 3rd percentile for weight and height, being 15kg and 118cm at 20 years of age, and had typical facial features with a beaked nose, micrognathia and mid-facial cyanosis. In 1985 he had a cerebrovascular accident causing a left hemiparesis following which he was hypertensive requiring treatment. He was taking propanolol, hydrallazine and aspirin, with nifedipine used intermittently to combat emergency elevation of his blood pressure. In 1987 (age 14) he suffered from recurrent transient episodic loss of speech, thought to be cerebrovascular in origin, though carbamazepine was also started at that time for possible epileptogenic events. He had shown partial recovery from his hemiparesis, but also become functionally blind due to vascular complications. From 1992 onwards DP was receiving injections of a fibroblast growth factor, based on preliminary studies suggesting a beneficial effect from this treatment in children with hypoxic brain damage (Aguilar et al. 1993). DP had a large myocardial infarct in March 1996, and died from complications 24 hours later.
4.1.4.2 HG1 Progeria

These cells were purchased from the Corriel Cell Repository, National Institute of Ageing, U.S.A. (Reference no. AG10578). At the time of biopsy this was a male aged 16 years and 5 months, with short stature, alopecia, age spots on his skin and coxa valga. He had suffered 4 heart attacks. A clinical diagnosis of Hutchinson-Gilford progeria had been made. Fibroblast cultures were established from a skin biopsy taken anti-mortem on 27.6.89 frozen down after 6 population doublings. Further details were not available.

4.1.5 A549 B2Neo.ρ0 cells

These cells were the generous gift of Dr. Ian Holt, Ninewells hospital, Dundee. It is an immortal cell line derived from a human (male) lung carcinoma. MtDNA depletion has been achieved by prolonged growth (6-8 weeks; >10 passages) in ethidium bromide at 50ng/ml (Desjardins et al. 1985). The cells are also transfected with a gene conferring neomycin resistance. These ρ0 cells are dependant on pyruvate and uridine supplemented media. The parental (+mtDNA) A549 cells were also available.

4.1.6 Controls

Control fibroblasts cultures were established from non-smoking volunteers aged 9 to 77 years who had no significant medical history, and were on no medication. None had any family history of blindness or other neuromuscular disease. Two control lymphoblast lines were purchased from the European Collection of Animal Cell Cultures, Porton Down, UK (RPMI 7666 and NC37). A further lymphoblast line was available to us from a 2 month old child with epidermolysis bullosa (junctional type; Dr. Paul Rutland, Institute of Child Health, Great Ormond Street), but no known mitochondrial disease. This was included as a control.
4.2. Tissue culture

4.2.1 Tissue culture mediums

Fibroblast growth medium:
- Dubelco's modified Eagles medium (DMEM) without pyruvate, with high glucose (4.5g/litre), and containing 2mM glutamine, penicillin 50units/ml, streptomycin 50mg/ml, fetal calf serum 10%, 0.2mM uridine 50mg/ml and 1mM sodium pyruvate.

Lymphoblast growth medium: RPMI 1640 medium with additions as above.

Selection medium: as for fibroblast medium but lacking pyruvate and uridine, and with additional geneticin 300μg/ml.

All cells were grown at 37°C, 92% O₂ and 8% CO₂.

All chemicals and plates were from Life Technologies Ltd. except pyruvate and DMSO which were from Sigma Chemical Co.

4.2.2 Establishment of primary fibroblast cultures

All tissue culture work was carried out under sterile conditions in Class 2 microbiological safety hoods. In all cases informed consent by the individual (or parent) and approval from the local ethics committee had been granted. Partial thickness pinch skin biopsies were taken from the inner arm under local anaesthetic and aseptic conditions but without prior cleaning of the skin with alcohol/iodine compounds as this was found to interfere with subsequent cell growth. The sample was placed straight into chilled fibroblast medium and stored at 4°C for a maximum of 24 hours. The skin was diced into 0.5mm³ pieces and placed in 35mm non-coated tissue culture plates (5-10/plate) with 0.5ml growth medium. After 4-7 days when the fragments could be seen to be adhering to the plate a further 1.5ml medium was added. Medium was then changed once a week until a halo of fibroblasts could be seen extending 3-5 mm around the fragments. The cells were then harvested with trypsin (as
below) and placed in a fresh 35mm plate until confluent. Passaging onto larger plates (60mm to 100mm) was undertaken and cells frozen down at passage numbers 3 - 4.

4.2.3 Culture maintenance

Fibroblasts, A549 and A549p0 cells were grown on non-coated 100mm plastic culture dishes and passaged when confluent. (0.5-1 x 10^6 cells). One passage was counted as splitting one plate onto two. Fibroblast growth media was removed twice a week and plates washed with phosphate buffered saline (PBS) before replacing with fresh media. For passaging and harvesting cells plates were washed twice in PBS and incubated with 1ml of trypsin (0.1% in versene 1:5000) at 37°C for 2-4 minutes until all cells were easily dislodged by gentle tapping of the plate. The trypsin was inactivated by re-suspending the cells in fresh fibroblasts growth media.

Lymphoblasts were grown in 260ml/80cm² plastic flasks. Media was topped up 2 - 3 X a week, and if discoloured (yellow) changed by pelleting the cells at 350g_{max} for 5 minutes and resuspending in fresh media. Culture were split when healthy clumps formed (~ 5-10 X 10^5 cells/ml).

4.2.4 Cell Freezing

One confluent plate of cells was harvested and pelleted by centrifugation at 350g_{max} for 10 minutes. The pellet was resuspended in 0.3-0.5mls of filter-sterilized growth medium containing 10% dimethyl-sulphoxide (DMSO), and placed in 1ml cryotubes. Slow cooling of cells was achieved by placing vials in polystyrene racks in polystyrene boxes at -70°C overnight before storage in liquid nitrogen.
4.2.5 Clonal cultures

A confluent plate of low passage number cells was harvested, spun at 350g_{max} for 10 minutes, and the cell pellet resuspended in a known volume (50 - 200µl) of medium. 10 - 40µl was removed and diluted with 10µl tryptan blue to identify dead cells. Cells were counted with a haemocytometer. The cell suspension was then diluted with growth medium to 2 cells/ml, and 0.25-1ml of the cell suspension placed in each of 96 wells (4 X 24 well plates). The plates were examined at 1-3 day intervals and wells containing only a single cell marked. Cell colonies derived from these single cells were fed weekly with fibroblast growth medium containing fungizone 1µg/ml, until the clonal colonies approached 50% confluency. These were harvested onto 35mm plates and thereafter onto larger plates before freezing and analysis.

4.2.6 Growth Curves

Harvested cells were counted as above and 1 X 10^5 cells placed on each of eight 100mm plates/cell line. Four plates were grown in normal growth medium and four in selection medium. One of each set was then harvested every 2-3 days, the number of cells counted and plotted against time.

4.2.7 Enucleation (Veomett, 1982)

Plastic 2cm coverslips were punched out from the bottom of 35mm tissue culture dishes (Medical Engineering, Royal Free Hospital) and sterilized with ultraviolet light. These were collagen coated by incubation in 35mm dishes with collagen (0.5ml in 50ml PBS) at 37°C for at least 2 hours. Twenty-four hours prior to enucleation 5 x 10^4 cells were plated on each of 5-10 coverslips and grown in normal medium overnight or until just confluent. The coverslips were placed, cells down, in 5mls of pre-warmed cytochalasin B in DMEM (10µg/ml, no other additives) in an autoclaved centrifuge tube. (Stock
cytochalasin B, Sigma Chemical Co., 5mg/ml in DMSO stored at -20°C). The cells were then spun in a pre-warmed Kontron Centrikon T-124 centrifuge at 35-37°C at 25-30,000g\textsubscript{max} for 19-22 minutes in order to obtain at least 90% enucleation as determined by fixing cells from trial coverslips in ethanol:acetic acid 3:1 and staining with Mayer's haematoxylin. After centrifugation the cells were placed in fresh growth medium and incubated at 37°C for 20 minutes to allow them to recover. For each set of fusions a number of trials were carried out in order to ascertain the optimum conditions for enucleation of that cell line. The actual conditions used for each cell line are given in the results sections.

4.2.8 Cell fusion (Norwood and Zeiger, 1992)

3 - 6 \times 10^5 neomycin resistant \rho^0 A549 cells were placed with 2mls of medium on top of the cytoplasts in a 35mm dish and incubated together for 4 hours. Solutions required for fusion were prepared on the day in sterile containers pre-warmed to 37°C. After the co-incubation period 8 sterile beakers were prepared as follows in the tissue culture hood:

- 3 x DMEM (no additives)
- 1 x 50% w/v autoclaved polyethylene glycol (PEG) molecular weight 1,000g in DMEM with 10% DMSO.
- 3 x DMEM with 10% DMSO, filter sterilized.
- 1 x fibroblast growth medium

The coverslip with both cytoplasts and \rho^0 cells adherent was washed 3 times in DMEM and touch dried against a microscope slide. The cells were then exposed to the fusogen (PEG) with slight agitation for 1 minute, then washed 3 times in DMEM with 10% DMSO and finally in normal growth medium. The coverslip was then placed back into fibroblast medium in a culture dish and incubated for 24 hours. The cells were then harvested with trypsin and plated at low cell density (1 coverslip divided onto 2-4 100mm plates) in selection media.
This was changed twice a week and plates regularly checked for growth of colonies. The \( p^0 \) cells are dependent on pyruvate and uridine, and non-enucleated/non-fused fibroblasts killed by the geneticin present. Hence any growing colonies developing represent fused cells containing the A549 nucleus with mtDNA derived from the enucleated fibroblast line. At 14-28 days growing colonies were isolated within a sterile ring (cut from the top of micro-centrifuge tubes) dipped in silicone grease and harvested individually with trypsin onto 35mm plates. These clonal colonies were grown in selection medium for a further 5 passages before initial analysis or freezing.

4.2.9 Mycoplasma testing of cell lines

Cells were screened for infection with mycoplasma species by Dr. M. Cleeter, (Dept of Clinical Neuroscience, Royal Free Hospital, London) using a commercially available kit. (Boehringer Mannheim mycoplasma PCR ELISA kit). The assay is based on the amplification of a mycoplasma-specific DNA sequence by the polymerase chain reaction, and subsequent detection of the amplicon by an enzyme linked immuno-sorbence assay, and was conducted according to the manufacturer's instructions. Treatment of cells where indicated was undertaken with 20\( \mu \)g/ml of Ciprofloxacin (Bayer) added to fresh media on three occasions over a 10 day period. A further 10 days was allowed for recovery before re-testing of the cells or experimental work.

4.3 DNA analysis

4.3.1 Cell lysis

1 x \( 10^6 \) to \( 10^7 \) cells were harvested with trypsin, washed twice with 5ml PBS, resuspended in 200\( \mu \)l PBS and stored in a 1.5ml eppendorf tube at -20\( ^\circ \)C prior to lysis. For cell lysis the cells were thawed and washed three times with 0.6ml of TE buffer (10mM Tris, 1mM EDTA, pH 8.0) and repelleted by
centrifugation at 15,000 g_{\text{max}} for 5 minutes. The final pellet was incubated for 20 minutes at 55°C in 0.2ml cell lysis buffer containing 50mM KCl, 20mM Tris-Cl (pH 8.3), 2.5mM MgCl₂, 0.45% (v/v) Tween 20 and 0.45% (v/v) Nonidet P40 with 80μg proteinase K. 0.1ml of sterile double distilled water (ddH₂O) was then added to this incubation solution and the cells were incubated at 90°C for a further 10 minutes before storage at -20°C until PCR analysis was carried out.

4.3.2 DNA extraction

DNA was extracted from 1-3 X 10⁶ cultured cells using the Nucleon I DNA extraction kit (Scotlab) according to the manufacturers instructions.

4.3.3 Polymerase chain reaction (PCR) DNA amplification

The PCR amplification reaction contained primers at a concentration of 1μM each, 10mM Tris-Cl (pH 9.0 at 25°C), 50mM KCl, 0.1% triton X-100, 1.5mM MgCl₂, 0.2mM each dNTP, and 10μl of cell lysate or 100ng of extracted DNA in a final volume of 50μl.

The PCR amplification cycle was 4 minutes at 96°C after which 1.25 units of Taq DNA polymerase (Promega UK) was added, followed by 30 cycles of denaturing (1 minute 92°C), annealing (1 minute-X°C) and extension (X minutes 72°C), with a final extension of 10 minutes. All amplifications were performed on a Hybaid Omnigene PCR unit. Primers were synthesized by British Biotecnology Ltd. Details of primer sequences, annealing temperatures and extension times are given in Table 3.
<table>
<thead>
<tr>
<th>DNA mutation</th>
<th>forward primer bp</th>
<th>5' - 3' sequence</th>
<th>reverse primer bp</th>
<th>5' -3' sequence</th>
<th>annealing temp</th>
<th>extension time</th>
<th>product size(bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11,778 LHON</td>
<td>11,091 - 11,110</td>
<td>CAG CCA CAG</td>
<td>11,191 - 11,172</td>
<td>GTA AGC CTC TGT TGT CAG AT</td>
<td>55°C</td>
<td>1 min</td>
<td>1,110</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CAC TAA TCA TA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14,484 LHON</td>
<td>14,464- 14,483</td>
<td>AGT ATA TCC</td>
<td>14,664- 14,645</td>
<td>GCT TTG TTT TTG TTG AGT GT</td>
<td>55°C</td>
<td>1 min</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AAA GAC AAC GA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3,460 LHON</td>
<td>2,928- 2,947</td>
<td>CCT AGG GAT</td>
<td>3,558- 3,539</td>
<td>TAG AAG AGC GAT GGT GAG AG</td>
<td>59°C</td>
<td>1 min</td>
<td>630</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AAC AGC GCA AT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4,160 LHON</td>
<td>4,021- 4,040</td>
<td>ACT ACA ATC</td>
<td>4,210- 4,161</td>
<td>TAA GTA ATG CTA GGG TGA GTG GTA</td>
<td>55°C</td>
<td>1 min</td>
<td>190</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TTC CTA GGA AC</td>
<td></td>
<td>GGA AGT TTT TTC ATA CCA GGT GTA TC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6,852 Hinf1</td>
<td>6,490- 6,514</td>
<td>TCC TCT TCT TCC</td>
<td>7,779- 7,756</td>
<td>CAG ACG GTT TCT ATT TCC TGA GCG</td>
<td>57°C</td>
<td>1.5 mins</td>
<td>1,289</td>
</tr>
<tr>
<td>site</td>
<td></td>
<td>CAG TCC TAG CTG C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>wild-type (non-Deleted)</td>
<td>8,171- 8,190</td>
<td>TGC TCT GAA</td>
<td>9,630- 9,611</td>
<td>CTC CTG ATG CGA GTA ATA CG</td>
<td>58°C</td>
<td>1.5 mins</td>
<td>1,459</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ATC TGT GGA GC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot;common&quot; deletion</td>
<td>8,196- 8,215</td>
<td>ACA GTT TCA</td>
<td>13,524- 13,505</td>
<td>CGA TGA TGT GGT CTT TGG AG</td>
<td>55°C</td>
<td>1 min</td>
<td>351</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TGC CCA TCG TC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4.3.4 Restriction enzyme digestion

For identification of LHON associated mtDNA mutations 8μl of PCR product was incubated with restriction enzymes as detailed in Table 4, in a final volume of 10μl. For confirmation of heteroplasmy in selected 3,460 clonal cell lines efficient digestion of the PCR product was confirmed by including an additional mtDNA fragment bearing a separate *HinI* 1 restriction site in the incubation. In these cases a mtDNA fragment including the 6,852bp *HinI* 1site was amplified from the cell lysates, and 10μl of the resultant PCR product incubated, together with 10μl of 3,460 PCR product, with the restriction enzyme.

4.3.5 Agarose gel separation

All agarose gels were made with TAE buffer (final concentrations: 0.04M Tris-acetate, 1mM EDTA, pH 8.0) with 5μl/100ml ethidium bromide. 10μl of PCR/restriction product was loaded in each well with 2μl 6X loading buffer (0.25% bromophenol blue in 40% sucrose) and the gel run for 2-4 hours at 20-35 volts until adequate separation was achieved. Details of agarose concentrations are given in Table 5. Running markers were supplied by Promega:

- φ174 low molecular weight marker-*Hae* III (11 fragments 72bp-1,353bp)
- φ174 low molecular weight marker-*Hinf* 1 (20 fragments 24bp-726bp)

Electrophoresed gels were viewed and photographed under ultra-violet light.
Table 4. Restriction enzyme digestion details for identification of LHON mtDNA mutations.

<table>
<thead>
<tr>
<th>Mutation &amp; site change</th>
<th>Restriction enzyme</th>
<th>Recognition sequence</th>
<th>Reaction buffer</th>
<th>Reaction conditions</th>
<th>Digestion products (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11,778 G to A site gain</td>
<td>Mae III (Boehr.Manh.)</td>
<td>5' /GTNAC 3' 3' CANTG/ 5'</td>
<td>20mM Tris-HCl pH 8.2, 275mM NaCl, 6mM MgCl₂, 7mM β-mercaptoethanol</td>
<td>3 units 55°C, 4 hrs</td>
<td>wild-type: 560, 255, 228, 57, mutant: 560, 228</td>
</tr>
<tr>
<td>14,484 T to C site loss</td>
<td>Mbo I (Strategene)</td>
<td>5' /GATC 3' 3' CTAG/ 5'</td>
<td>10mM KOAc, 10mM MgAc, 25mM Tris-Ac pH 7.6, 10μg/ml BSA, 0.5mM β-mercaptoethanol</td>
<td>5 units 37°C, 1 hr</td>
<td>wild-type: 180, mutant: 200</td>
</tr>
<tr>
<td>3,460 A to G site loss</td>
<td>Hinl I (Strategene)</td>
<td>5' GPu/CGPyC 3' 3' CPyGC/PuG 5'</td>
<td>25mM Tris-HCl pH 7.7, 50mM NaCl, 10μg/ml BSA, 10mM MgCl₂, 10mM β-mercaptoethanol</td>
<td>3.5 units 37°C, 1 hr</td>
<td>wild-type: 530, 100, mutant: 630</td>
</tr>
<tr>
<td>4,160 T to C site loss</td>
<td>BstXI (Sigma)</td>
<td>5' CCA(N5)/NTGG 3' 3' GGTN/(N5) ACC 5'</td>
<td>50mM Tris-HCl pH 7.9, 10mM MgCl₂, 100mM NaCl, 1mM dithiothreitol</td>
<td>2 units 55°C, 2 hrs.</td>
<td>wild-type: 143, 47, mutant: 190</td>
</tr>
<tr>
<td>common deletion</td>
<td>Xba I (NBL Ltd.)</td>
<td>5'T/CTAGA 3' 3'AGATC/T 5'</td>
<td>6mM Tris-HCl pH 7.9, 150mM NaCl, 6mM MgCl₂, 6mM β-mercaptoethanol</td>
<td>4 units 37°C, 2 hrs.</td>
<td>mutant: 251, wild-type: 100</td>
</tr>
</tbody>
</table>

hr(s) = hour(s); bp = base pairs. Suppliers are given in brackets. Boehr.Manh. = Boehringer Mannheim
Table 5. Agarose gels for separation of restricted PCR products as listed

<table>
<thead>
<tr>
<th>mtDNA products</th>
<th>% agarose</th>
<th>agarose supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>11,778</td>
<td>3</td>
<td>Metaphor</td>
</tr>
<tr>
<td>14,484 and 4,160</td>
<td>4</td>
<td>Metaphor</td>
</tr>
<tr>
<td>3,460</td>
<td>1.2</td>
<td>Sigma</td>
</tr>
<tr>
<td>wild-type</td>
<td>1</td>
<td>Sigma</td>
</tr>
<tr>
<td>common deletion</td>
<td>2</td>
<td>Sigma</td>
</tr>
</tbody>
</table>

4.3.6 Densitometric analysis of DNA gels (3,460 LHON clones)

Completed digestion in heteroplasmic clones was confirmed by HinI restriction of 10µl of 630bp PCR product from each cell line together with 10µl of a 1,289bp PCR product which included an additional HinI site, resulting in 362bp and 927bp fragments. For the estimation of mutant load in heteroplasmic 3,460 fibroblasts (at p = 10) electrophoresed DNA gels were analysed on a Kontron Vidas AT image analysis system with a monochrome Sony 77CS 768X512 pixel CCD camera, using the Imaging Associated Vidas 2.5 Gel analysis software package (Mr. J. Muddle, Dept of Clinical Neurosciences, Royal Free Hospital). To ensure the optical density of scanned samples were linear with respect to load, a standard curve was prepared using the 630bp product. Restricted PCR products (neat, 1:1, and 1:3 dilutions) were electrophoresed, scanned and after subtraction of the background image, and the percentage mutant versus wild-type bands for each sample estimated.

4.3.7 Estimation of common deletion levels

DNA extracted from a patient with CPEO known to have 80% deleted mtDNA in muscle on southern blotting (Prof. A. E. Harding, Dept. of Neurogenetics, Institute of Neurology, London) was used as a positive control and a standard curve constructed using serial dilutions with wild-type mtDNA. PCR amplification of these samples and of DNA extracted from control
fibroblasts was carried out in parallel on three separate occasions, and 10μl of PCR product from each sample electrophoresed on agarose gels. A total of 100ng DNA was used for each 50μl PCR reaction. A visual estimation of the approximate level of mtDNA bearing the common deletion in each control subject was made with reference to the standard curve.

4.3.8 Analysis of mtDNA in DP fusions

DNA extracted from DP-A549ρ0 cells was characterized by Dr. R. Chalmers, University Department of Clinical Neurology, Institute of Neurology, Queen Square, London. Sequence analysis revealed that the A549 parental cells had a D-loop polymorphism involving loss of one CA repeat in a string of 5 between bp 514 and 523 (L-strand). An H strand primer (3' 516-535 5') with an A for T mismatch at 517 introduced an Alu I restriction site in the A549 line. PCR products generated using this primer and an L strand primer (3' 371-390 5') yielded a product of 163bp, which was cleaved into 20 and 143bp by Alu I. PCR products from the A549 cells, DP fibroblasts and DP-A549ρ0 cybrid clone 3 and 4 were digested with Alu I at 37°C for 4 hours and run with undigested product on a 3.2% agarose/ethidium bromide gel.

4.3.7 Genomic DNA fingerprinting

DNA fingerprinting was carried out on DNA extracted from cells by Cellmark Diagnostics, Abingdon, Oxfordshire, U.K. Five to 8μg of DNA was digested overnight with Hinf I (New England Biolabs) and analysed on 0.7% agarose gels in TBE (Tris-borate-EDTA) containing 0.5μg/ml ethidium bromide. Gels were run at 75V until a 2.3 kb marker fragment had migrated 20cm. Gels were treated with 0.25M HCl for 15 minutes, 0.5M NaOH for 30 minutes and 0.5M Tris-HCl pH 7.5/ 3M NaCl for 30 minutes. Blotting onto Hybond N membrane (Amersham) and UV fixation were carried out according to the manufacturers instructions. Prehybridization was performed in 0.5M Na2HPO4, 0.1% SDS for 20 minutes at 50°C followed by hybridization of multilocus
probes (MLP) 33.15 and 33.6 (Jeffreys et al. 1985) in 0.5M Na$_2$HPO$_4$, 0.1% SDS, 1% Casein (Hammarsten) for 20 minutes at 50°C. Washing was carried out at 50°C in 80mM Na$_2$HPO$_4$, 0.1% SDS for 10 minutes and repeated. Two final washes at room temperature were carried out in 100mM Maleic acid, 150mM NaCl pH 7.5 for 10 minutes. Labelled bands were detected with chemiluminescence (Lumi-Phos 530) and autoradiography at 30°C using Amersham Hyper film MP.

4.4. Mitochondrial preparations

4.4.1 Preparation of mitochondrial enriched fractions (MEFs)

MEFs were prepared from 20-25 x 10cm confluent plates of fibroblasts, 10-12 confluent plates of A549 derived cells, or 4-6 80ml flasks of lymphoblasts (1 - 1.5 x 10$^7$ cells), by homogenization and differential centrifugation (Ragan et al. 1987). Harvested cells were washed 3 times in PBS and the pellets frozen overnight at -70°C. The pellets were thawed and resuspended in 2 ml of ice cold homogenization buffer (10mM Tris-Cl 1mM EDTA and 0.25M sucrose pH 7.4). Each sample was homogenized on ice in Potter-type glass/Teflon homogenizers (Uniform, Jencons) for 20 strokes at 1000 rpm, and spun at 1,500g$_{\text{max}}$ for 10 minutes at 4°C. The resultant post-nuclear supernatant (PNS) was collected into a fresh tube. Homogenization of the residual pellet and centrifugation were repeated a further 2 times, and the combined PNS spun at 1500g$_{\text{max}}$ for 10 minutes and any residual pellet discarded. The PNS was spun at 10,000g$_{\text{max}}$ for 12 minutes at 4°C on a Kontron Centrikon T-124 centrifuge to obtain a MEF. This pellet was resuspended in 300-1000μl of homogenization buffer, snap frozen in liquid nitrogen and stored at -70°C for 3-7 days prior to biochemical analysis.
4.4.2 Mitochondrial enrichment efficiency

In order to assess the reproducibility of MEF preparations, a selection of cell pellets were processed as follows: after thawing, the cells were initially suspended in 1ml of homogenization buffer, and a 100µl aliquot removed and stored for later biochemical analysis. The remaining suspension was made up to 2mls and an MEF prepared from this as described above. Complex II/III activity was then assayed in both the cell suspension and the MEF, as detailed in 4.5.3, and mitochondrial enrichment calculated as follows:

\[
\text{enrichment} = \frac{\text{complex II/III specific activity in MEF}}{\text{complex II/III specific activity in cell suspension}}
\]

4.5 Respiratory chain enzyme analysis

MEFs from patients and controls were analysed simultaneously in batches. MEFs were freeze-thawed three times prior to analysis. Assays were performed on either Hitachi U3210 or Kontron 940 dual-beam spectrophotometers at 28°C in a final volume of 1ml. Each enzyme was assayed in triplicate and values accepted if they were within +/- 10% of their means. Unless otherwise stated MEFs were prepared from each subject on at least 3 separate occasions. Chemicals were from Merck (UK) and Sigma Chemical Company.

4.5.1 Citrate synthase (CS) assay (Coore et al. 1971)

CS is a mitochondrial matrix enzyme which was used as an indicator of mitochondrial mass in enriched preparations as it has not been found to be altered in disease states. Respiratory chain activities were expressed as CS ratios to correct for variation in purity in the mitochondrial preparations. The appearance of DTNB-CoA was measured at 412nm in a reaction mixture containing 100mM Tris-HCl pH 8.0, 200µM acetyl CoA, 0.1% Triton X-100 and 200µM 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB). The reaction was initiated
with 100µM oxaloacetate (OAA) and the activity calculated using a molar extinction coefficient of 13.6 X 10^3.

4.5.2 NADH-Ubiquinone oxidoreductase (Complex I) assay (Ragan et al. 1987)

4.5.2.1 Calculation of ubiquinone-1 (CoQ₁) concentration

CoQ₁ was the generous gift of the Eisai Chemical Company, Tokyo, Japan. The CoQ₁ was diluted in ethanol and the absorbence at 275nm measured. An excess of sodium borohydride was added to the reference cuvette to reduce ubiquinone to ubiquinol, and the resultant change in absorbence recorded. The volume of ubiquinone needed for a final cuvette concentration of 50µM was calculated using a molar extinction coefficient of 12.25 X 10^3.

4.5.2.2 Complex I assay

Complex I activity was measured by following the oxidation of NADH at 340nm in a reaction mixture containing 20mM K-phosphate buffer pH 7.2, 10mM MgCl₂, 150µM NADH, 1mM KCN, 2.5mg/ml bovine serum albumin (BSA, fatty acid free) and 50µM CoQ₁. Initial and rotenone (10µM) sensitive rates were calculated using a molar extinction coefficient for NADH of 6.81 x 10^3, modified to take account of the contribution of CoQ₁ to the absorption at 340nm.

For studies on rotenone sensitivity complex I was assayed as above using 0-10µM rotenone added in 10µl of ethanol. For studies on the diphenyleneiodonium (DPI) inhibitable rate samples were pre-incubated in the complex I reaction mixture, lacking NADH, with 30µM DPI at 30°C for 10 minutes. The reaction was initiated with NADH.
4.5.3 Succinate cytochrome c oxidoreductase (Complex II/III) assay

(King, 1971)

Complex II/III activity was assayed by monitoring the appearance of reduced cytochrome c at 550nm in a reaction mixture containing 0.1M K phosphate buffer pH 7.4, 0.3mM EDTA (di K), 0.1mM cytochrome c, 2.5mg/ml BSA, 20mM succinate and 1mM azide. Samples were pre-incubated with succinate and azide for 15 minutes at 28°C before initiation of the reaction by addition to the remaining reagents. After 20 minutes antimycin A (20μM) was added. Complex II/III activity is that which is sensitive to antimycin A and was calculated using a molar extinction coefficient of 19.2 × 10^3.

4.5.4 Cytochrome Oxidase (Complex IV) assay (Wharton and Tzagoloff, 1967)

4.5.4.1 Preparation of reduced cytochrome c

A 1% cytochrome c solution (Boehringer Mannheim, horse heart crystallized, salt free) was prepared in 0.01 M K-Phosphate buffer, pH 7.0 and reduced fully by adding a pinch of L-ascorbic acid. This was confirmed by adding 50μl of the reduced cytochrome c (R.cyt.c) to 950μl of K-Phosphate buffer in 2 cuvettes and looking for any further absorbence change at 550nm with the addition of 10μl saturated ascorbic acid solution to the sample cuvette. When >95% reduced the cytochrome c was aliquoted into 25cm sections of dialysis tubing (Medicell International Ltd., size 1) and dialysed for 8-24 hours in K-phosphate buffer at 4°C. At intervals the % reduction was checked as previously described. To ensure complete absence of ascorbate one of two cuvettes with 50μl of R.cyt.c in 950μl of buffer was fully oxidized with 0.1M KFeCN and the absorbence monitored at 550nm to confirm no evidence of ongoing reduction in the presence of KFeCN. This would imply the continuing presence of ascorbic acid, and the need to continue dialysis. These checks were repeated until the R.cyt.c was >95% reduced with no evidence of residual ascorbate. R.cyt.c was stored in 1ml aliquots at -70°C.
4.5.4.2 Calculation of R.cyt.c concentration

50μl of R.cyt.c was added to 0.01M K-Phosphate buffer pH 7.0 in a final volume of 1ml. The change in absorbence at 550nm caused by addition of 0.1M KFeCN was noted. 50μM R.cyt.c should result in an absorbence change of 0.96 when oxidized, so:

\[
\frac{0.96}{\text{change in absorbence}} \times 50 = Y \text{ ml R.cyt.c needed}
\]

This was checked by using Y ml R.cyt.c in the same reaction and looking for an absorbence change of 0.96.

4.5.4.3 Complex IV assay

Complex IV activity was assayed by following the oxidation of R.cyt.c at 550nm in a reaction mixture containing 0.01M K-phosphate buffer pH 7.0 and 50μM R.cyt.c. The reference cuvette was oxidized using 0.1M KFeCN to achieve a starting absorbence of 0.96 +/- 5%. This assay is first order with respect to cytochrome c so the pseudo-first order rate constant k was calculated and activity expressed as k/minute/mg protein.

4.5.5 General details

Stock solutions of all buffers, acetyl CoA, Triton X-100, BSA, cytochrome c, succinate, EDTA, rotenone and antimycin A were made in advance and stored at -20°C. Rotenone and antimycin A were also protected from light. Solutions of DTNB, OAA, KCN, azide, NADH and KFeCN were made fresh and kept on ice. All samples were kept on ice after freeze-thawing. Statistical calculations and linear regression analysis were carried out using a computer software statistics package ("Instat", Graphpad Software) unless otherwise stated.
4.5.7 Protein determination

The protein concentration of each sample was measured in quadruplicate by the method of Lowry et al. (1951), calculated from a standard curve of 0 - 80μg BSA using linear regression analysis. Sample was diluted to a final volume of 1ml with water. This was incubated at room temperature with 5mls of a solution containing 2%(w/v) Na₂CO₃, 0.4% (w/v) NaOH, 0.01% (w/v) CuSO₄ and 0.02%(w/v) NaK-tartrate. After 20 minutes 0.5mls of 50% (v/v) Folin's phenol reagent was added. After a further 45 minutes the absorbence of each sample was read at 750nm.

4.6 Measurement of ATP synthesis in permeablized cells
(Wanders et al. 1993)

4.6.1 Substrate incubation and digitonin permeabilization

One confluent plate (0.5-1.0 X 10⁶ cells) of each cell line was used for each incubation. The requisite number of cells were harvested with trypsin, washed 3 times in PBS and the pellet resuspended in 5 - 10mls of incubation buffer containing 150mM KCl, 25mM Tris-Cl (pH 8.0), 2mM EDTA, 10mM KH(PO)₄, 1mM ADP and 0.1%(w/v) BSA, to achieve an estimated cell count of 0.5-1.0 X 10⁶/ml. Cells in a small aliquot of this suspension were counted, and the remainder split into 1ml aliquots. Cell permeabilization was achieved with digitonin (98%, Sigma Chemical Co.) at a final concentration of 80μg/ million cells. Respiratory substrates (pyruvate and malate or glutamate and malate) were added at a final concentration of 10mM to each aliquot. The reaction was allowed to proceed at 37°C for 20 minutes before termination with of 0.5M perchloric acid and incubation on ice for 15 minutes. Each sample was centrifuged at 15,000g_max for 2 minutes at 4°C, and the supernatant removed into a fresh tube and neutralized by the addition of 3M K₂CO₃/0.5M triethanolamine. Any remaining precipitate was again pelleted and discarded. Samples were stored at -70°C for a maximum of one week before ATP assays were carried out.
The optimum concentration of digitonin was determined by adding 0-250µg/million cells to control incubations with pyruvate and malate and measuring ATP production at each concentration. The optimum reaction time was similarly determined by terminating reactions at 5-40 minutes in a control cell line. This data will be presented in the results (Chapter 6.1). For experiments looking at rotenone sensitivity rotenone was added in 5µl ethanol to achieve a final concentration of 0-5µM, prior to the addition substrates. The inhibited values were compared to those of incubations with 5µl ethanol alone to correct for any artefact produced by the ethanol.

4.6.2 ATP measurement

ATP generation was measured by quantitating the NADH formed in the following coupled reaction:

\[
\text{Hexokinase} \\
\text{Glucose + ATP} \rightarrow \text{Glucose-6-Phosphate + ADP} \\
\]

\[
\text{Glucose-6-phosphate} \quad *\text{G6PDH} \quad \text{Gluconate-6-phosphate} \\
+ \text{NADP} \rightarrow \text{Gluconate-6-phosphate + NADH} \\
*\text{Glucose-6-phosphate dehydrogenase} \\
\]

The reaction mixture contained 168mM Tris-Cl buffer (pH 8.0), 1.68mM MgCl₂, 0.168mM EDTA (K+), 0.1mM glucose, 20mM NADP, 2 units of G6PDH and 0 - 320µl of sample. The reaction was initiated with the addition of 2.5units of hexokinase in a final volume of 2.5mls and monitored on a Perkin-Elmer LS50 luminescence spectrophotometer. A fresh standard curve of ATP (0 - 30nmols) was prepared on each day, from which ATP generated by samples was calculated using linear regression analysis. Samples were assayed in triplicate on the day and results accepted if within +/-10% of their mean. The results were then expressed as nmols ATP/million cells.
4.6.3 Concentration of ATP stock

In order to accurately quantify the amount of ATP being added for the standard curve a stock ATP solution (10mM ATP-MgCl₂, pH 7.0; stored at -70°C) was quantified on a Hitachi U3210 dual beam spectrophotometer at 340nm and the actual amount of NADP oxidized calculated according to Beer-Lambert’s Law: $\Delta \text{Absorbence} = \varepsilon \times \Delta \text{concentration(M)} \times \text{pathlength}$

where pathlength = 1 and $\varepsilon = 6.22 \times 10^3$ for NADH.

60-140µl of 1mM ATP (60-140nmols) were added to a final volume of 1.25mls consisting of 168mM assay buffer (as above), 10mM glucose, 0.2mM NADP and 2 units G6PDH. The reaction was initiated with 2.5units hexokinase and allowed to proceed for 10 minutes. All ATP values as derived from the fluorimeter were corrected to the actual concentration of stock ATP determined in this way.

4.7. Analysis of complex I proteins

4.7.1 SDS-PAGE of mitochondrial fractions

Mitochondrial enriched fractions were prepared from tissue culture cells as previously described (chapter 4.4) and stored at -70°C with the addition of protease inhibitors (1mM pepstatin A, 1mM leupeptin and 1mM phenyl-methane-sulfonyl-fluoride, Sigma Chemical Co.). 10-12mg of protein was solubilized at 37°C for 5 minutes in sample buffer containing 10mM Tris-Cl, pH 8.0, 1mM diNa-EDTA, 2.5%(w/v)SDS, 0.1% (w/v) bromophenol blue and 5% (v/v) mercaptoethanol, before loading onto a 0.75mm thick 15% SDS-polyacrylamide minigel (Hoeffer “mighty-tall” system). Bovine heart complex I (Dr. J. M. Cooper, Royal Free Hospital) and a pre-stained rainbow molecular weight marker (14.3-200kDa Amersham) were also run on the same gel. Gels were run at 100V for 1 hour, then at 200V until the lowest marker was 1.5cm from the bottom.
Gel recipes: (f.c.) Separating  Stacking

<table>
<thead>
<tr>
<th></th>
<th>(f.c.) Separating</th>
<th>Stacking</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-Cl pH 8.7</td>
<td>375mM</td>
<td>-</td>
</tr>
<tr>
<td>Tris-Cl pH 6.8</td>
<td>125mM</td>
<td></td>
</tr>
<tr>
<td>30% acrylamide (1% Bis)</td>
<td>15% w/v</td>
<td>5% w/v</td>
</tr>
<tr>
<td>SDS</td>
<td>0.15% w/v</td>
<td>1% w/v</td>
</tr>
<tr>
<td>10% APS (ammonium persulphate)</td>
<td>0.034%w/v</td>
<td>1% w/v</td>
</tr>
<tr>
<td>TEMED (tetramethylethylene diamine)</td>
<td>0.05% v/v</td>
<td>0.3% v/v</td>
</tr>
</tbody>
</table>

Running Buffer: 0.8M Glycine, 25mM Tris, 0.1% SDS

4.7.2 Western Blotting for complex I proteins

Proteins were blotted onto a polyvinylidene difluoride (PVDF-Millipore) membrane by electro-elution (100V for 1.5 hours) in Towbins buffer containing 25mM Tris-Cl, 192mM glycine and 20% methanol (v/v), pH 8.3 (Towbin et al. 1979). Dried membranes were rinsed in methanol and then blocked for 2 hours in 0.3%(v/v) Tween 20/PBS (PBS-Tween) containing 10%(w/v) milk powder and 1%(w/v) ovalbumin. Polyclonal primary antibodies raised in rabbit against bovine complex I were available in the department (Dr. J. M. Cooper, Clinical Neurosciences, Royal Free Hospital). After two PBS-Tween washes, blots were incubated overnight with primary antibodies diluted in PBS-Tween containing 1%(w/v) ovalbumin as listed:

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Target protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP α J</td>
<td>1:100</td>
<td>Iron-protein fraction</td>
</tr>
<tr>
<td>39 α E</td>
<td>1:100</td>
<td>39kDa subunit</td>
</tr>
<tr>
<td>Cx 1 α D</td>
<td>1:100</td>
<td>Complex I holoenzyme</td>
</tr>
</tbody>
</table>

Blots were washed three 3 times in PBS-Tween, and then incubated for 2 hours with the secondary antibodies (goat anti-rabbit IgG with alkaline
phosphatase conjugate, Sigma Chemical Co.), diluted 1:3,000 in PBS-Tween. Following four PBS-Tween washes and two PBS washes labelled proteins were detected by incubation with nitroblue-tetrazoleum (300µg/ml) and 5-bromo-4-chloro-3-indolyl-phosphate (150µg/ml) in a buffer containing 100mM Tris-HCl, 100mM NaCl and 5mM MgCl₂, pH 9.5.

4.7.3 Staining of protein gels

Gels were stained overnight with 0.1% (w/v) Phastgel blue R250 dye in 40% Methanol and 10% glacial acetic acid (v/v), rinsed in methanol destain (40% methanol, 10% Glacial acetic acid, v/v) for 3 hours, and finally rinsed in water overnight.

4.8 Analysis of complex IV proteins

4.8.1 Protein extraction from tissue culture cells (Di Monte et al. 1993)

A single plate of confluent cells (0.5-1 X 10⁶) was harvested with a brief trypsinization, inactivated with media and then washed thoroughly in PBS (Chapter 4.2.3). The cell pellet was resuspended in 1 ml of ice-cold PBS containing 1mM protease inhibitors as for complex I proteins (Chapter 4.7.1). The cells were spun in 1.5ml eppendorf tubes at 750g_max for 2.5minutes at 4°C and the supernatant discarded. This was repeated and the final pellet resuspended in the PBS/protease inhibitors solution with the addition of 1.5% dodecylmaltoside. This suspension was shaken at 4°C for 30 minutes with occasional agitation to disrupt any precipitation forming. The suspension was then spun at 15,000g_max for 20mins at 4°C, and the final supernatant containing the cellular proteins decanted into a fresh 1.5ml eppendorf tube and stored at -70°C until use. Protein concentrations were determined by the Lowry method (Chapter 4.5.7).
4.8.2 SDS-Urea PAGE of protein extracts

Proteins were separated on 15% SDS-PAGE gels containing 6M urea. Running and stacking gels were prepared in BioRad Mini-Protean II gel systems. Equal amounts of protein for each sample were incubated at 37°C for 30 minutes in sample buffer containing 15mM Tris pH 6.8, 4% (w/v) SDS, 2% (v/v) glycerol and 0.1% (w/v) bromophenol blue. 10 - 15μg of protein/well was then loaded onto the stacking gel. A Gibco pre-stained low molecular weight marker (3 - 43k) was also loaded. The gel was run at 100V for 30-45mins, and then at 200V until the front was seen to run off the bottom.

Gel recipes: (for 2 X 1.5mm mini-gels) (f.c)Separating Stacking

<table>
<thead>
<tr>
<th></th>
<th>Seperating</th>
<th>Stacking</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-Cl pH 8.8</td>
<td>345mM</td>
<td>-</td>
</tr>
<tr>
<td>0.5M Tris pH 6.8</td>
<td>-</td>
<td>50mM</td>
</tr>
<tr>
<td>30% acrylamide</td>
<td>15% w/v</td>
<td>6.5% w/v</td>
</tr>
<tr>
<td>(0.8% Bis)</td>
<td>0.092% w/v</td>
<td>0.05% w/v</td>
</tr>
<tr>
<td>10% SDS</td>
<td>6M</td>
<td>6M</td>
</tr>
<tr>
<td>Urea</td>
<td>6M</td>
<td>6M</td>
</tr>
<tr>
<td>10% APS (ammonium persulphate)</td>
<td>0.044% w/v</td>
<td>0.05% w/v</td>
</tr>
<tr>
<td>TEMED (tetramethylethylene diamine)</td>
<td>0.06% v/v</td>
<td>0.06% v/v</td>
</tr>
<tr>
<td>Running Buffer: 0.8M Glycine, 25mM Tris, 0.1% SDS</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4.8.3 Western Blotting of Complex IV proteins

Transfer of proteins to PVDF-Millipore membrane was undertaken as in 4.7.2. Prior to antibody labelling of blotted proteins the membrane was blocked in 10% (w/v) low fat milk in PBS-Tween for 2 hours at room temperature, and washed twice in PBS-Tween. Monoclonal mouse antibodies to the N-terminal region of human porin (Konstantinova et al. 1995) were supplied by Dr. F. P. Thinnes, (Max Planck Insitut, Göttingen, Germany), and diluted 1:10,000 in PBS-Tween. Monoclonal mouse anti-IgG antibodies were kindly supplied by Dr. J. W. Taanman, Department of Clinical Neurosciences, Royal Free Hospital,
(Capaldi et al. 1995). Blots were incubated overnight in the primary antibody at the following dilutions in PBS-Tween:

<table>
<thead>
<tr>
<th>Hybridoma cell line</th>
<th>Subunit target</th>
<th>dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1D6-E1-E8</td>
<td>I</td>
<td>1:15,000</td>
</tr>
<tr>
<td>12C4-F12</td>
<td>II</td>
<td>1:18,000</td>
</tr>
<tr>
<td>20E8-C12</td>
<td>IV</td>
<td>1:1,000</td>
</tr>
</tbody>
</table>

After three washes in PBS-Tween antigen-antibody complexes were incubated for 2 hours with horseradish peroxidase conjugated goat anti-mouse IgG antibodies (BioRad), followed by three washes PBS-Tween and one in PBS. Bound antibody was detected by chemi-luminescence (Dupont Renaissance kit) and autoradiography with ECL Film (Amersham).

4.9 Mitochondrial in vitro translation studies on cultured fibroblasts

4.9.1 Preparation of 35S labelled mitochondrial fractions from cultured cells

Two confluent 100mm plates of fibroblasts for each cell line were incubated at 37°C overnight (18 hours) in normal growth medium containing 40μg/ml chloramphenicol. After thorough washing in growth medium, plates were incubated with 4mls of Met-Cys-Medium (Sigma) supplemented with 5% dialysed fetal calf serum, glutamine, penicillin and streptomycin as in 4.2.1. Emetine was added to each plate at a final concentration of 100μg/ml, and to one plate from each sample chloramphenicol was also added (200μg/ml). These cells were preincubated at 37°C for 15 minutes before the addition of 0.925 MBq/ml of 35S Easytag express protein labelling mix, containing 35S labelled cysteine and methionine (Dupont). After a pulse of 4 hours with frequent mixing a chase of 18 hours was carried out using normal growth medium. Each plate was harvested individually using a cell scraper, washed
three times in PBS and frozen at -70°C for 30 minutes. Mitochondrial fractions were isolated from each sample using homogenization and differential centrifugation as in 4.4 except that cells were homogenized in only 0.5mls of buffer, and the final low speed spin was omitted in order to minimize sample loss. The mitochondrial pellet was then resuspended in 20μl of sample buffer containing 62.5mM Tris-HCl pH 6.8, 2% SDS, 10% sucrose, 10mM dithiothrirotol and 0.001% bromophenol blue, and proteins dissociated at 37°C for 5mins before pelleting insoluble material by centrifugation.

4.9.2 Scintillation counting of labelled products

5μl of each sample adsorbed to a glass micro-fibre filter (Whatman, UK) was placed in ice-cold 10%(w/v) trichloracetic acid (TCA) with 0.5%(w/v) methionine for 1.5 hours. Each disc was then boiled for 15 minutes in the 10%TCA/methionine solution, and then boiled for 15 minutes in 5%TCA/methionine, rinsed in ethanol and air-dried at room temperature for 30 minutes before placing in 10mls of Ultima-gold scintillant (Canberra-Packard). Scintillation counting was undertaken for 5 minutes / sample on a Beckman LS5000 CE scintillation counter.

4.9.3 SDS-Urea-PAGE translation products

Mitochondrial samples of equivalent counts were loaded onto a 6M Urea 16.5% SDS-Polyacrylamide gel using the recipe given in Chapter 4.8.2 and a Hoeffer "mighty-tall" gel system, together with a 14C labelled low molecular weight rainbow marker (Amersham), and run at 70 volts until adequate separation of marker was achieved (overnight). Radioactivity was detected using fluorography with 2,5-diphenyloxazole in acetic acid (Skinner and Griswold, 1983). Gels developed in this way were dried under vacuum at 80°C and exposed to pre-flashed Fuji medical X-ray film for 7 - 21 days.
4.10 Presentation of results and discussion

The findings in LHON and in HGPS have been presented independently, in Parts 3 and 4 respectively. In both, separate chapters each consist of a brief introduction, followed by the results and relevant discussion. The findings from each of the chapters are then discussed more broadly in final chapters for each condition, where future work is also considered.
Part 3.
Leber's hereditary optic neuropathy
results and discussion

Chapter 5. MtDNA analysis and preliminary MRC function in LHON

Introduction:

Previous studies of MRC function in LHON subjects carrying either the 11,778 mutation, or less commonly the 3,460 mutation, have to date yielded inconsistent results as reviewed in Chapter 2.6.2. A complex I defect has been demonstrated in 3,460 platelets (Howell et al. 1991a; Smith et al. 1994) and lymphocytes (Majander et al. 1991), but the situation in 11,778 LHON is less clear, and there are no reports concerning families with the 14,484 mutation except in the presence of an additional 4,160 mutation (Parker et al. 1989), or where this has not been excluded (Oostra et al. 1995). Functional data from all three primary mutations have not been reported by any single group.

The aim of the work presented in this Chapter was to compare MRC enzyme activities in subjects with each of the three primary LHON mutations with those of controls. Lymphoblasts from an 11,778 family were available to us, and fibroblasts obtained from 3,460 and 14,484 families.

5.1 Mitochondrial enrichment

In order to assess the reproducibility of mitochondrial preparations randomly selected preparations from a variety of cell lines were assessed as described in Chapter 4.4.1. The mean enrichment factor was 3.17 +/- 1.21 (n = 10). To compensate for the variable contribution of mitochondrial protein to the total protein concentration in MEFs, respiratory chain enzyme results are expressed as citrate synthase (CS) ratios as well as specific activities (nmols/min/mg protein).
5.2.1 DNA analysis

Restriction enzyme analysis of PCR-amplified mtDNA from cell lysates confirmed the presence of homoplasmic 11,778bp G to A mutation in lymphoblasts cultures established from the clinically affected male proband and two clinically unaffected cousins in the maternal line (Figure 3). The G to A 11,778 mutation results in an additional Mae III restriction site. The 255bp product seen in the control has been digested into bands of 127bp and 128bp in the 11,778 subjects, visible as a single band on the gel shown. The 4,160bp, 3,460 or 14,484 mutations were not detected in these three subjects (data not shown), and this family are known not to harbour the 15,257 or 13,708bp mutations (Prof. A. E. Harding, Institute of Neurology, London).

Figure 3. Mae III restricted PCR products from lymphoblast cell lysates of one control (C), and three 11,778 LHON family members separated on an ethidium bromide stained 3% agarose gel. M = θ Hinf 1 marker

bp
560 -
255 -
225 -
127/8 -
C IV3 IV10 IV11
5.2.2 MRC enzyme analysis

Enzyme assays were performed on MEFs prepared in triplicate from cultured lymphoblasts from the three 11,778 subjects, and three control lymphoblasts lines as detailed in Chapter 4.5. No significant differences in MRC enzyme activities were seen between the two groups, whether expressed as specific activities or CS ratios (Table 6). A non-parametric t-test modified for small sample numbers was used for statistical analysis (Parker, 1979).

5.3 14,484 LHON

5.3.1 DNA analysis

Restriction enzyme digestion of PCR-amplified mtDNA confirmed the presence of homoplasmic 14,484bp G to A mutation in fibroblast cultures established from the affected male proband, and his clinically unaffected sister and mother (Figure 4). The T to C 14,484 mutation results in a loss of the Mbo1 restriction site seen in the control. The 200bp product in the control has digested into 180bp and 20bp products, although the 20bp fragment cannot be seen, whereas a 200bp product persists in the 14,484 patients. None were found to posses the 4,160bp mutation (data not shown) and this family are known not to have either the 15,257, 3,460 or 11,778bp mutations (Prof. A. E. Harding, Institute of Neurology, London).
Table 6. MRC enzyme activities MEFs from 11,778 LHON lymphoblasts

A: Specific activities

<table>
<thead>
<tr>
<th></th>
<th>CS</th>
<th>NADH:CoQ&lt;sub&gt;1&lt;/sub&gt; R rot. insens.</th>
<th>NADH:CoQ&lt;sub&gt;1&lt;/sub&gt; R rot. sens.</th>
<th>SCcR</th>
<th>COX k/min/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (n=3)</td>
<td>118.4 +/- 41.5</td>
<td>7.0 +/- 2.5</td>
<td>16.1 +/- 3.0</td>
<td>56.7 +/- 11.9</td>
<td>2.45 +/- 0.20</td>
</tr>
<tr>
<td>11,778 LHON (n=3)</td>
<td>96.0 +/- 20.2</td>
<td>6.9 +/- 0.9</td>
<td>13.73 +/- 0.86</td>
<td>33.7 +/- 4.2</td>
<td>2.31 +/- 0.20</td>
</tr>
</tbody>
</table>

B: Citrate Synthase Ratios

<table>
<thead>
<tr>
<th></th>
<th>NADH:CoQ&lt;sub&gt;1&lt;/sub&gt; R rot. insens. (X100)</th>
<th>NADH:CoQ&lt;sub&gt;1&lt;/sub&gt; R rot. sens. (X10)</th>
<th>SCcR (X10)</th>
<th>COX (X100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (n=3)</td>
<td>6.62 +/- 0.34</td>
<td>1.38 +/- 0.07</td>
<td>5.03 +/- 1.31</td>
<td>2.30 +/- 0.08</td>
</tr>
<tr>
<td>11,778 LHON (n=3)</td>
<td>7.63 +/- 0.47</td>
<td>1.51 +/- 0.26</td>
<td>3.73 +/- 0.30</td>
<td>2.54 +/- 0.41</td>
</tr>
</tbody>
</table>

MRC enzyme activities on 11,778 LHON patients (age 38 - 40 years) and controls (range 2 months - 36 years). A: specific activities are expressed as nmol/min/mg protein unless otherwise stated. B: data from A expressed as a ratio with CS (citrate synthase) activity. NADH:CoQ<sub>1</sub> R = NADH coenzyme Q<sub>1</sub> reductase (complex I); Mitochondrial complex I activity is taken as that which is sensitive to rotenone (rot.sens.); SCcR = succinate cytochrome c reductase (complex II/III); COX = cytochrome oxidase (complex IV). Values are mean +/- SD.
Figure 4. Mbo 1 restricted PCR products from fibroblast cell lysates of one control (C), and three 14,484 LHON family members separated on an ethidium bromide stained 4% agarose gel. M = φ 174 Hinf 1 marker

5.3.2 MRC enzyme analysis

Enzyme analysis was performed on triplicate mitochondrial preparations from cultured fibroblasts of the three 14,484 LHON subjects, and compared to the results from 7 age matched controls (Table 7). Statistical analysis using the Mann-Whitney U-test revealed no significant differences between the groups.
MRC enzyme analysis on 14,484 LHON patients (age 39 - 75 years) and healthy controls (range 31 - 77 years). Mitochondrial complex I activity is taken as that which is sensitive to rotenone. A: specific activities are expressed as nmol/min/mg protein unless otherwise stated. B: data from A expressed as a ratio with CS activity. Values are mean +/- SD.

### A: Specific activities

<table>
<thead>
<tr>
<th></th>
<th>CS</th>
<th>NADH:CoQ₁ R</th>
<th>NADH:CoQ₁ R</th>
<th>SCcR</th>
<th>COX</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rot. insens.</td>
<td>rot. sens.</td>
<td>rot. insens.</td>
<td></td>
<td>k/min/mg</td>
</tr>
<tr>
<td>Controls (n=7)</td>
<td>96.1 +/- 22.7</td>
<td>13.5 +/- 4.8</td>
<td>14.0 +/- 4.3</td>
<td>43.4 +/- 16.9</td>
<td>1.66 +/- 0.30</td>
</tr>
<tr>
<td>14,484 LHON (n=3)</td>
<td>91.9 +/- 11.9</td>
<td>14.6 +/- 3.3</td>
<td>14.2 +/- 2.1</td>
<td>38.5 +/- 3.7</td>
<td>1.67 +/- 0.25</td>
</tr>
</tbody>
</table>

### B: Citrate Synthase Ratios

<table>
<thead>
<tr>
<th></th>
<th>NADH:CoQ₁ R</th>
<th>NADH:CoQ₁ R</th>
<th>SCcR</th>
<th>COX</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rot. insens. (X10)</td>
<td>rot. sens. (X10)</td>
<td>(X10)</td>
<td>(X100)</td>
</tr>
<tr>
<td>Controls (n=7)</td>
<td>1.49 +/- 0.76</td>
<td>1.36 +/- 0.09</td>
<td>4.25 +/- 0.57</td>
<td>1.86 +/- 0.26</td>
</tr>
<tr>
<td>14,484 LHON (n=3)</td>
<td>1.61 +/- 0.37</td>
<td>1.63 +/- 0.46</td>
<td>4.24 +/- 0.79</td>
<td>1.78 +/- 0.36</td>
</tr>
</tbody>
</table>
5.4 3,460 LHON

5.4.1 DNA analysis

Restriction enzyme digestion of PCR amplified mtDNA from cell lysates (fibroblasts) is shown in Figure 5. At passage number 5 the male proband (IV1) was found to be homoplasmic for the 3,460bp G to A mutation. The 3,460 G to A mutation results in a loss of a *HinI* restriction site, and persistence of the 630bp PCR product, compared to the 530bp product seen in the control (and a 100bp product not seen). Both the mother (III2) and brother (IV2) appeared heteroplasmic, as a faint band of 530bp product was visible, representing wild-type mtDNA. The proportion of 3,460 mutant: wild-type mtDNA did not change significantly at higher passage numbers (p 12 and 17, Figure 5) in these subjects. The 14,484 and 11,778 and 4,160bp mutations were not detected in any of this family and they are known not to possess the 15,257 or 13,708 mutations (Prof. A. E. Harding, Institute of Neurology, London).

5.4.2 MRC function in 3,460 LHON

Analysis of MRC enzymes performed on MEFs prepared from cultured fibroblasts from the three 3,460 subjects revealed a significant and specific decrease in complex I activity when compared to the results from 9 age matched controls (Table 8). This was evident whether expressed as a specific activity (mean decrease 53%; p<0.01) or a CS ratio (mean decrease 57% defect; p<0.01). No significant difference in CS, complexes II/III or IV were observed. The complex I activity in the homoplasmic proband was slightly lower than that of his heteroplasmic mother or brother but these differences were not significant (Table 9).
Figure 5. *Hin*I restricted PCR products from fibroblasts lysates of one control (C) and three 3,460 family members (*IV*₁, *IV*₂, and *III*₂) separated on an ethidium bromide stained 1.2% agarose gel. The passage number of the fibroblasts at the time of DNA analysis is indicated below the subjects. M = 0 174 *Hae* III marker.
MRC enzyme analysis on 3,460 LHON patients (age 23 - 46 years) and healthy controls (range 21 - 50 years). Mitochondrial complex I activity is taken as that which is sensitive to rotenone. A: activities are expressed as nmols/min/mg protein unless otherwise stated. B: data from A expressed as a ratio with CS activity. Values are means +/- SD. * p < 0.01 (Mann Whitney U-test)

### A: Specific activities

<table>
<thead>
<tr>
<th></th>
<th>CS</th>
<th>NADH:CoQ₁ R rot. insens.</th>
<th>NADH:CoQ₁ R rot. sens.</th>
<th>SCcR</th>
<th>COX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (n=9)</td>
<td></td>
<td>94.5 +/- 20.0</td>
<td>13.8 +/- 5.0</td>
<td>14.9 +/- 4.7</td>
<td>43.0 +/- 15.5</td>
</tr>
<tr>
<td>3,460 LHON (n=3)</td>
<td>106.6 +/- 28.0</td>
<td>14.2 +/- 4.4</td>
<td><strong>6.9 +/- 0.8</strong></td>
<td>47.0 +/- 5.7</td>
<td>1.92 +/- 0.14</td>
</tr>
</tbody>
</table>

### B: Citrate Synthase Ratios(+/-SD)

<table>
<thead>
<tr>
<th></th>
<th>NADH:CoQ₁ R rot. insens. (X10)</th>
<th>NADH:CoQ₁ R rot. sens. (X10)</th>
<th>SCcR (X10)</th>
<th>COX (X100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (n=9)</td>
<td>1.50 +/- 0.66</td>
<td>1.55 +/- 0.35</td>
<td>4.50 +/- 0.70</td>
<td>2.01 +/- 0.23</td>
</tr>
<tr>
<td>3,460 LHON (n=3)</td>
<td>1.35 +/- 0.13</td>
<td><strong>0.67 +/- 0.01</strong></td>
<td>4.55 +/- 0.10</td>
<td>2.05 +/- 0.13</td>
</tr>
</tbody>
</table>
Table 9. Complex I results from individual 3,460 subjects.

<table>
<thead>
<tr>
<th>Subject</th>
<th>IV1</th>
<th>IV2</th>
<th>III2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific activity</td>
<td>7.45 +/- 3.64</td>
<td>6.07 +/- 2.06</td>
<td>7.31 +/- 3.06</td>
</tr>
<tr>
<td>CS ratio (X10)</td>
<td>0.56 +/- 0.08</td>
<td>0.76 +/- 0.19</td>
<td>0.68 +/- 0.20</td>
</tr>
</tbody>
</table>

Values are the means +/- SD of three preparations from each subject. The specific activity is expressed as nmols/min/mg protein.

5.4.3 DNA analysis in 3,460 clonal cell lines

The restricted PCR products from clonal fibroblast lines (\( p = 5 \) after cloning) derived from LHON subjects III\( _2 \) and IV\( _2 \) are shown in Figure 6. Eight of 12 clones analysed from III\( _2 \), and 23 of 28 clones from IV\( _2 \) were homoplasmic for the 3,460 mtDNA mutation based on visual assessment of the ethidium bromide stained gels. Three clones from IV\( _2 \) contained only wild-type mtDNA (clones 4, 10 and 24). The remaining clones were heteroplasmic.

5.4.4 MRC function related to % mutant load in 3,460 LHON

Sufficient material for complex I, CS and mtDNA analysis was obtained from only 6 clonal lines after 10 passages. The percentage mutant load in these cell lines was estimated by densitometry of restricted PCR products described in 4.3.5. The range in which the amount of PCR product loaded on the gel was linear with respect to optical density, was established by construction of a standard curve as shown in Figures 7A and B. The restricted PCR products from the clones are shown in Figure 8. Of the 6 clonal lines only III\( _2 \) clone 2 remained heteroplasmic at passage no.10, with 73% mutant. The percentage mutation in the other clones is shown in Table 10.
Figure 6. *Hind*I 1 restricted PCR products from 3,460 fibroblast clones at p = 5. M = θ 174 Hae III marker; H = water blank; C = control. The clones are represented by their numbers, labelled sequentially as they were harvested from the initial 24-well plates.
Figure 7A. Standard curve of serial dilutions of the 630bp PCR product from 3,460 fibroblasts on an ethidium bromide stained 1.2% agarose gel. The volume ($\mu$l) of undiluted product loaded in each lane is shown.

Figure 7B. Standard densitometry curve of serial dilutions of the 630bp PCR product from 3,460 fibroblasts
Figure 8. *Hin*l 1 restricted PCR products from selected 3,460 fibroblast clones at p = 10, as used for biochemical analysis. The restricted product from IV\(^1\) (primary culture) is also shown.

Table 10. Densitometric assessment of percentage mutation in restricted PCR products from 3,460 fibroblast clones.

<table>
<thead>
<tr>
<th>Fibroblasts clone</th>
<th>% 3,460 mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>III(^2) clone 2</td>
<td>73%</td>
</tr>
<tr>
<td>IV(^2) clone 4</td>
<td>0</td>
</tr>
<tr>
<td>IV(^2) clone 8</td>
<td>100</td>
</tr>
<tr>
<td>IV(^2) clone 12</td>
<td>0</td>
</tr>
<tr>
<td>IV(^2) clone 21</td>
<td>0</td>
</tr>
<tr>
<td>IV(^2) clone 28</td>
<td>100</td>
</tr>
</tbody>
</table>
The relationship between percentage 3,460 mutation and complex I:CS ratio is shown in Figure 9. Clonal lines with 0% (undetected) 3,460 mutation (IV₂ clones 4, 12, and 21) had a mean complex I:CS ratio of 0.12. The mean complex I:CS ratio from two clones homoplasmic for the 3,460 mutation (IV₂ clones 8 and 28) was 66% lower at 0.04. The persistently heteroplasmic clone (III₂ clone 2) had a complex I:CS ratio intermediate between these values (0.07).

Figure 9. Complex I activity in MEFs from fibroblast clones heteroplasmic for the 3,460 LHON mutation. Points represent the mean of triplicate assays from a single mitochondrial preparation from IV₂ clone 8 (○), clone 28 (■), clone 12 (▼) and clone 21 (♦), and from two separate preparations from IV₂ clone 4(#) and III₂ clone 2 (▲).
5.5 Discussion: MtDNA and respiratory chain enzyme analysis in LHON

5.5.1 11,778 LHON

Reports of MRC function in 11,778 LHON have to date given rise to conflicting messages. Normal electron transfer through complex I has been demonstrated in muscle (Larsson et al. 1991) and lymphocytes (Majander et al. 1991). These studies included measurements of rotenone sensitive NADH:CoQ$_1$ reductase and NADH:cytochrome c reductase, as well as NADH ferricyanide reductase, representing the proximal part of the holoenzyme. In contrast Smith et al. (1994) reported a mild (20%) decrease in complex I activity (rotenone sensitive NADH:CoQ$_1$ reductase) in platelets homoplasmic for the 11,778bp mutation. This defect was only apparent when the smoking habits of the subjects were taken into consideration. Smith et al. (1993) had previously shown smoking to decrease complex I activity in platelet preparations.

Although none of the patients or controls in this study were smokers, there are insufficient numbers to draw firm conclusions on complex I activity. Electron transfer through complex I appears normal in the three 11,778 subjects, but a mild defect cannot be excluded from this limited study.

In contrast enzymatic analysis of fragmented mitochondria, functional assessments of intact mitochondria have given repeatedly abnormal results in 11,778 patients. Lymphoblasts homoplasmic for the 11,778bp mutation have exhibited a 75% decrease in the oxidation of NAD-linked substrates by intact mitochondria compared to controls (spectrophotometric analysis; Majander et al. 1991), and polarographic measurements of MRC function in muscle have also shown a 50% deficient with complex I linked substrates compared to controls (Larsson et al. 1991). Furthermore $^{31}$P-magnetic resonance spectroscopy (MRS) studies on brain and muscle have also indicated there is abnormal oxidative metabolism in subjects with the 11,778 mutation (Cortelli et al. 1991; Barbiroli et al. 1995): all subjects in these two studies, both clinically affected and unaffected, had evidence of a decreased energy reserve in brain
compared to 20 controls (reduced phosphocreatine/inorganic phosphate (PCr/Pi) ratio), and although basal muscle measurements were sometimes within normal limits (50 controls), all had low PCr recovery after exercise, considered a better indicator of MRC function. An explanation for the apparent discrepancy between the results from enzyme studies and functional assessments on intact mitochondria has been proposed by Esposti et al. (1994): this study demonstrated that in mitochondria with the 11,778bp mutation, there was altered the affinity of complex I for the ubiquinone substrate, and for the inhibitor used in most enzyme studies, rotenone. Esposti et al. propose that in vivo the arginine to histidine substitution resulting from the 11,778 mutation, might result in reduced stability of the ubisemiquinone intermediate which would rapidly dismutate and interact with oxygen to form free radicals that may contribute to further complex I damage. In vitro enzyme assays of NADH oxidation by exogenous ubiquinones might not detect this defect, if by implication the free radical damage was reversible, as the ubiquinone acceptor undergoes rapid auto-oxidation under these conditions. In intact mitochondria in vitro, integrated electron transport along the respiratory chain would still be impaired, as rapid auto-oxidation of ubisemiquinone also implies decreased efficiency in releasing the ubiquinol product by the mutant complex I affecting overall function. The existence of a ubisemiquinone intermediate has been supported by other studies (Vinogradov et al. 1995), though no direct evidence for excessive production of free radicals in LHON mitochondria has been reported.

Thus the probable conclusion in 11,778 LHON is that a MRC defect does exist though detection may vary according to the methodology used. The dilemma that remains however is that the perturbation of mitochondrial function observed in the studies discussed above, was observed in both clinically affected and unaffected carriers of the mutation. Indeed those subjects in whom in vivo deficiencies in muscle and brain metabolism have been demonstrated have no clinical evidence of CNS or muscle dysfunction, and in
some cases no evidence even of visual disturbance. Thus whilst the 11,778bp mutation may be linked to MRC dysfunction, the relevance of this in terms of disease mechanisms is unclear. Impaired respiratory chain function may be an innocent marker of some other pathogenetic process, albeit linked somehow to the mtDNA mutation. This will be further discussed after presentation of data on the other primary mutations in the final LHON Chapter (8).

5.5.2 14,484 LHON

The 14,484 mutation converts a moderately conserved methionine to valine in the hydrophilic loop of the complex I ND6 subunit (Mackey and Howell, 1992). This amino acid change has not been observed in control populations and does not require the co-existence of known secondary mutations for clinical penetrance (Obermaier-Kusser et al. 1994), so is now considered to be a primary mutation. This mutation accounts for 10-15% of all LHON cases (Riordan-Eva and Harding, 1995) and it is generally considered to be less deleterious with a better visual outcome (Brown et al. 1994; Riordan-Eva et al. 1995). The ratio of clinically affected subjects is more male biased than for the other primary mutations according to recent pedigree analysis (Riordan-Eva et al. 1995). Studies of respiratory chain function in 14,484 patients have previously been reported from the Queensland family first described by Wallace in 1970. Whilst exhibiting a severe complex I defect in platelet mitochondria (Parker et al. 1989), this family also harbour a mutation at position 4,160 (ND1; Howell et al. 1991b) and are clinically very atypical for LHON due to the presence of a severe central nervous system involvement including a childhood encephalopathy. A further report showing a 35% defect in complex I electron transfer, and a milder (20%) defect of complex I linked ATP synthesis in four affected LHON probands with the 14,484 mutation, did not give clinical details, and had not excluded the 4,160 mutation (Oostra et al. 1995). This study (section 5.3) has not demonstrated a complex I defect in 14,484 subjects. It was originally suggested that the CNS manifestations in the Queensland family
might be caused by the 4,160 mutation rather than the 14,484 LHON mutation (Howell et al. 1991b). The 4,160 mutation might also be primarily responsible for the complex I defect shown by Parker et al. in 1989. Failure to demonstrate a complex I defect in 14,484 subjects lacking the 4,160 mutation lends weight to this hypothesis.

Results from only three 14,484 subjects as presented here cannot exclude the presence of a mild complex I defect that might be detectable in larger series. It is also feasible that, as has been proposed for the 11,778bp mutation, an in vivo defect might be apparent using assessments on intact mitochondria which is not readily detectable with the methods used, though as the 14,484bp mutation affects a different subunit identical functional defects might not be expected. As with the 11,778 mutation both clinically affected and unaffected carriers were indistinguishable in terms of MRC function assessed in this way, which will be further discussed in Chapter 8.

5.5.3 3,460 LHON

The observed 57% decrease in complex I activity in fibroblast mitochondria possessing the 3,460bp LHON mutation is consistent with previous studies (Howell et al. 1991a; Majander et al. 1991; Smith et al. 1994). This biochemical defect has previously been attributed to the 3,460bp mutation which is postulated to impair normal electron transfer either by affecting a critical binding site in complex I, or by altering the conformation of the final holoenzyme complex (Howell et al. 1991a). A 78% decrease in rotenone sensitive NADH:ubiquinone oxidoreductase was demonstrated in platelet mitochondria from four 3,460 subjects compared to 41 age matched controls by Smith et al. (1994). Howell et al. (1991a) found a 73% decrease in the platelet/white blood cell fraction of 5 subjects compared to 5 controls, and Majander et al. (1991) an 80% decrease in EBV-transformed lymphoblasts from two 3,460 LHON patients compared to a single control line. That the defect demonstrated in fibroblasts in this study is slightly less severe may reflect the
presence of some wild-type mtDNA in 2 of these 3 subjects, whereas all the previously reported cases were homoplasmic for the mutation. An apparent correlation between mutant load and the severity of the biochemical defect (Chapter 5.4.4) would lend support to this hypothesis.

Biochemical data on a broader range of heteroplasmic clones would help to clarify this point further. However many of the clonal lines initially established grew very slowly, and showed macroscopic evidence of senescence, followed by cell death. Some were lost before any DNA analysis could be carried out at p = 5. Those which survived to this level were mostly found to be homoplasmic, and all but one of those initially heteroplasmic had drifted towards homoplasmy by p = 10, the lowest passage number yielding sufficient mitochondria from biochemical analysis. That the mutant load in LHON families rapidly drifts towards homoplasmy through subsequent generations has been well documented (Harding et al. 1995; Ghosh et al. 1996), although in the more recent of these studies there was also one instance where there was a decrease in mutant load from parent to offspring. Some drift towards homoplasmy has also been observed in heteroplasmic 11,778 fibroblast clones in culture, though to a lesser extent (Matthews et al. 1995). This pattern is in contrast with other disease-causing mtDNA mutations where heteroplasmy is the usual finding both in tissues (Hammans et al. 1993) and at an intracellular level (Matthews et al. 1995). The factors that control mitochondrial genotype drift are not understood. Ghosh et al. (1996) concluded from their study, and on reviewing the literature on heteroplasmic LHON families, that although there was an overall tendency to increasing mutant load in successive generations, the segregation and replication of mtDNA molecules in LHON cells was subject to conditions of random drift. This is not supported by the clonal data presented in this thesis, although the number of clonal lines subject to sequential DNA analysis over increasing passages was limited. That cells and individuals carrying LHON mutations are predominantly homoplasmic for LHON mutations is in keeping with the hypothesis that the biochemical consequences of LHON
mutations are less deleterious to the cell than those found in other mitochondrial diseases.

A significant decrease in complex I activity associated with the 3,460 mtDNA mutation has now been documented in a total of 14 subjects in the studies presented above. However six of these subjects (III_2, IV_2 from this study, 2 from Smith et al. 1994 and 2 from Howell et al. 1991) are as yet clinically unaffected at ages ranging from 22 to 50 years. It is possible that they may yet develop visual loss, but also possible that as for the other primary mutations the biochemical defect in 3,460 LHON may not correlate with the clinical expression of the disease. In addition this is not a tissue specific defect as it is expressed in fibroblasts as well as peripheral blood cells with this mutation. This apparent widespread complex I defect contrasts with the tissue specificity of the clinical disease. This may in part reflect the low oxidative demands of fibroblasts and blood cells, but in tissues such as muscle and brain any functional defect may be expected to be of greater clinical significance, yet all 14 of the 3,460 subjects reported, both affected and unaffected, are clinically normal in respect of muscle and CNS function outside the visual pathways. The tissue specificity of the disease then remains unexplained.

This consistent complex I defect in 3,460 LHON contrasts to the normal activities in 14,484 LHON (Chapter 5.5.2), and the conflicting data in 11,778 LHON (Chapter 5.5.1). The severity of this complex I defect and its apparent widespread tissue distribution is perhaps surprising given the lack of correlation with clinical deficits both between subjects, and within individuals in whom a very tissue specific clinical effect is observed. Hence further characterization of the functional consequences of the 3,460bp LHON mutation was undertaken and will be detailed in Chapter 6. Furthermore this defect also provides a clearly defined functional marker which can be used to study the effects of a control nuclear environment on the biochemical expression of the 3,460 mutation, as nuclear genes have been implicated in disease expression in
LHON by pedigree analysis. These results will be presented and discussed in Chapter 7.

Finally the functional inconsistencies between the different LHON mutations, the failure of functional data to date to distinguish between clinically affected and unaffected family members, and the possible roles of mtDNA mutations in the pathogenesis of LHON will be discussed in Chapter 8.
Chapter 6: Functional and structural characterization of complex I in 3,460 LHON fibroblasts

Introduction

As discussed in Chapter 5.5, a significant decrease in complex I electron transfer activity has been consistently observed in tissues possessing the 3,460 LHON mutation. Despite this the tissues, and in some cases the individual subjects, in which this defect has been demonstrated are clinically unaffected. Previous studies of MRC function in 3,460 LHON (Majander et al. 1991; Howell et al. 1991a; Smith et al. 1994), have been confined to assessment of complex I electron transfer activity in fragmented mitochondria. Analysis of MRC function in intact mitochondria has not been reported in 3,460 LHON. This study reports further characterization of the functional consequences of the 3,460bp LHON mutation in cultured fibroblasts. ATP synthesis in permeabilized cells was analysed, and a more detailed assessment of the electron transfer properties of 3,460 complex I undertaken by inhibitor studies on MEFs prepared from cultured fibroblasts.

6.1 ATP synthesis in 3,460 LHON fibroblasts

6.1.1 Setting conditions for analysis of ATP synthesis by cultured fibroblasts

The conditions for the production of ATP were optimized in control and 3,460 fibroblasts as described in Chapter 4.6. A sample ATP standard curve is shown in Figure 10. A typical digitonin titration and time course are shown in Figures 11 and 12. Maximum ATP synthesis with complex I linked substrates was observed with digitonin concentrations ranging from 40 - 120µg/ million cells. A sharp fall in ATP synthesis was seen at concentrations above or below this. Using 80µg digitonin/million cells ATP synthesis with complex I linked substrates was linear with respect to time from 0 to 25 minutes, after which a plateau was reached. For all subsequent experiments fibroblasts were incubated with 80µg digitonin/million cells in a final volume of 1ml for 20
minutes. This represents the mid-point of optimal digitonin concentrations, and a time point within the linear portion of the time curve.

Figure 10. A sample ATP:fluorescence standard curve. ATP was measured by quantitating the generation of NADPH formed in the presence of hexokinase, glucose-6-phosphate dehydrogenase, glucose and NADP as described in Chapter 4.6.2. The concentration of the ATP stock dilution was determined as described in Chapter 4.6.3. The fluorescence values obtained for each of several quantities of ATP were plotted as shown to ensure linearity within the study range.
Figure 11. Digitonin titration of a sample fibroblast line, assessed by ATP synthesis in the presence of pyruvate and malate. The maximum ATP production in this cell line was 116nmols ATP/million cells /20 minutes.

Figure 12. Time course of ATP synthesis from a control fibroblast line with pyruvate and malate and 80μg digitonin / million cells. Means +/- standard deviation of three experiments are shown. Maximal ATP synthesis was 145nmols ATP/million cells.
6.1.2 ATP production by 3,460 LHON fibroblasts

ATP production with complex I-linked substrates was assessed in quadruplicate in fibroblasts from all three 3,460 patients, and in 3 - 7 fibroblast preparations from each of 7 age-matched controls (21 to 50 years). ATP production with complex II- or complex IV-linked substrates was also measured, though in fewer preparations and subjects. The results are shown in Table 11. In the 3,460 fibroblasts ATP synthesized in the presence of pyruvate and malate, glutamate and malate, succinate or ascorbate were not significantly different from control values. The mean values from each subject with complex I linked substrates have been plotted in Figure 13. Although the mean ATP synthesis with pyruvate and malate was lower in 3,460 patients this did not reach statistical significance (Mann Whitney U-test), and the values fell largely within the control range.

Table 11. ATP production by control and 3,460 permeabilized fibroblasts.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Cell line</th>
<th>pyruvate &amp; malate</th>
<th>glutamate &amp; malate</th>
<th>succinate &amp; rotenone</th>
<th>ascorbate &amp; TMPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>controls</td>
<td>(n)</td>
<td>177 +/- 40</td>
<td>183 +/- 26</td>
<td>127 +/- 17</td>
<td>65 +/- 14</td>
</tr>
<tr>
<td></td>
<td>(7)</td>
<td>(5)</td>
<td>(5)</td>
<td>(5)</td>
<td>(5)</td>
</tr>
<tr>
<td>3,460</td>
<td>(n)</td>
<td>146 +/- 12</td>
<td>182 +/- 27</td>
<td>115 +/- 1</td>
<td>78 +/- 14</td>
</tr>
<tr>
<td></td>
<td>(3)</td>
<td>(3)</td>
<td>(2)</td>
<td>(2)</td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as nmols ATP/million cells/20 minutes. Values are means +/- SD of at least triplicate preparations from each subject; n = number of subjects studied.
Figure 13. ATP production with complex I linked substrates (x-axis) in control and 3,460 LHON permeabilized fibroblasts. Means from 3 - 6 preparations from individual controls are depicted as open circles, and from 3,460 subjects as open squares. Each group mean is shown as a filled symbol with standard deviations, calculated from the combined values of each subjects.
6.1.3 Validation of the ATP synthesis assay in the presence of MRC defects

In order to examine the relationship between decreased electron transfer through complex I and the level of ATP synthesis detectable by this method, a complex I defect was produced in control MEFs by the addition of rotenone in a range of concentrations to the reaction mixtures and NADH:CoQ1 reductase measured spectrophotometrically as described in Chapter 4.6. ATP synthesis in the presence of complex-I linked substrates (pyruvate and malate) was then measured on permeabilized fibroblasts from the same subjects, inhibited by a range of rotenone concentrations. This demonstrated that ATP synthesis was inhibited in parallel to NADH:CoQ1 electron transfer with increasing rotenone concentrations (Figures 14 and 15). At 12-15nM rotenone, rotenone sensitive NADH:CoQ1 reductase activity was approximately 60% inhibited. At the same rotenone concentrations ATP production was also inhibited to a similar degree.

6.2 Rotenone sensitivity in 3460 LHON

Rotenone sensitivity curves of NADH:CoQ1 reductase activity were performed on MEFs from two control and two 3,460 LHON subjects in order to assess whether the decrease in rotenone sensitive NADH:CoQ1 reductase activity observed in 3,460 LHON (Chapter 5.4), might merely represent ineffective rotenone binding due to a change in the conformation of ND1. At low rotenone concentrations 3,460 complex I was less inhibited than in controls with the I50 ([rotenone] resulting in 50% maximal inhibition) estimated to be 37.4nM in 3,460 subjects as compared to 8.2nM in controls (Figure 16). However at concentrations above 100nM this difference was no longer apparent, both groups being similarly inhibited.

Rotenone sensitivity of ATP production with complex I linked substrates in 3460 cells (n = 3) was not significantly different from that of controls (n = 3), even at low rotenone concentrations (Figure 17)
Figure 14. Rotenone inhibition of mitochondrial NADH:CoQ₁ reductase activity (A), and of ATP synthesis with pyruvate and malate (B) in control fibroblasts.
Figure 15. Relationship between ATP synthesis (permeabilized fibroblasts) and mitochondrial NADH:CoQ₁ reductase activity (MEFs) in control fibroblasts. Data interpreted from Figure 14.
Figure 16. Rotenone sensitivity of mitochondrial NADH:CoQ$_1$ reductase activity in MEFs from control (circles) and 3,460 LHON (squares) fibroblasts. Points represent the mean +/- SD from single preparations on each of two subjects. Where not indicated SD values were not sufficiently large to plot. *The maximal complex I activity in 3,460 LHON (CS ratio 0.075) was approximately 60% lower than in controls (0.125).
Figure 17. Rotenone inhibition of ATP production with pyruvate and malate in controls (n = 3; circles) and 3,460 LHON (n = 3; squares) permeabilized fibroblasts. The mean +/- SD at each rotenone concentration is shown. Where not indicated SD values were not sufficiently large to plot. *maximal ATP synthesis in the 3,460 subjects was 136 nmols/million cells/20 minutes, and 158 nmols/million cells/20 minutes in controls.

6.3 DPI sensitive NADH:CoQ\textsubscript{1} reductase activity in 3460 LHON

To confirm that the complex I electron transfer defect in 3,460 LHON was not a consequence of the inability of rotenone to inhibit the mutant enzyme, DPI sensitive NADH:CoQ\textsubscript{1} reductase activity was measured in MEFs prepared from two controls and two 3,460 patient fibroblasts as described in Chapter 4.5.2. The mean DPI sensitive NADH:CoQ\textsubscript{1} reductase:CS ratio in
3,460 subjects (0.034) was 82% lower than that of the 2 controls (0.19). This confirming the presence of a significant complex I defect in 3,460 fibroblasts (Table 12).

Table 12. DPI sensitive NADH:CoQ₁ reductase activity in MEFs from 3,460 LHON and control fibroblasts.

<table>
<thead>
<tr>
<th></th>
<th>NADH:CoQ₁ R DPI insensitive</th>
<th>NADH:CoQ₁ R DPI sensitive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=2)</td>
<td>0.186; 0.224</td>
<td>0.183; 0.197</td>
</tr>
<tr>
<td>3460 LHON (n=2)</td>
<td>0.286; 0.158</td>
<td>0.028; 0.039*</td>
</tr>
</tbody>
</table>

Values given are means from each subject. *p<0.05, calculated using a modified t-test for small sample numbers (Parker, 1979).

6.4 NADH:CoQ₁ reductase enzyme kinetics

The activities of rotenone sensitive NADH:CoQ₁ reductase activity were determined on MEFs from 3 controls and one 3,460 patient using varying concentrations of NADH in the reaction mixture (Figure 18). The $V_{\text{max}}$ and $K_m$ for the controls and 3,460 subjects were estimated from Eadie-Hofstee plots (Dawes, 1980), but limited data was available for more than a single assay at each NADH concentration and an extensive study could not be undertaken. The mean $V_{\text{max}}$ (+/- SD) in the three controls, expressed as a CS ratio, was 0.130 +/- 0.022 compared to a $V_{\text{max}}$ of 0.041 in the 3,460 subject. Estimation of $K_m$ in the three controls did not give consistent results with values of $4.1 \times 10^{-6}$, $0.8 \times 10^{-6}$ and $13.6 \times 10^{-6}$M for each subject. The $K_m$ in the 3,460 subject was $3.46 \times 10^{-6}$M.
Figure 18. Kinetics of mitochondrial NADH:CoQ$_1$ reductase activity with respect to [NADH]. Points represent single results at each concentration of NADH in fibroblast MEFs from a single 3,460 LHON subject (IV$_1$; open circles), and 3 control subjects (filled circles).

6.5 Mitochondrial protein studies in 3,460 LHON

6.5.1 Western blotting for complex I proteins.

Western blots were prepared from MEFs from the fibroblasts of 2 controls and two 3,460 LHON subjects (IV$_1$ and IV$_2$) as described in Chapter 4.7. Human heart mitochondrial fractions and purified bovine complex I were also studied. No differences in the pattern of antibody binding was observed between patient and control MEFs, using primary antibodies raised to bovine complex I. In each blot the bands have been identified on the basis of their relative mobilities. The results using an antibody directed against the Iron-protein fraction of complex I are shown in Figure 19. Subunits from human samples have slightly different mobility compared to the bovine complex I, but
the pattern in human samples from both 3,460 and control subjects is the same. The 18kDa band is missing in the bovine preparation. This may be a result of the complex I purification procedure, or it may not be a complex I protein. The antibody against the 39Kda subunit cross reacts with an 18kDa protein in both human and bovine samples (Figure 20). The 39kDa band is clearly visible in the human samples, but on this occasion was not seen in the bovine complex I lane. On other occasions using fresher complex I samples the bovine 39kDa band was clearly seen. The western blot using an antibody raised against bovine complex I holoenzyme is shown in Figure 21. Subunits in the human heart mitochondria preparation (H) are more clearly labelled than in the less pure fibroblast samples, but again no consistent difference between controls and patients is seen.

**Figure 19.** Western blot using a polyclonal antibody against the IP fraction of bovine complex I. M kDa = molecular weight marker positions; H=human heart mitochondria (control subject); B=bovine complex I. Other lanes are MEFs of fibroblasts from two controls (C) and two 3,460 subjects (IV₁ and IV₂).
Figure 20. Western blot using a polyclonal antibody against the 39kDa subunit of bovine complex I. M kDa = molecular weight marker positions; H=human heart mitochondria (control subject); B=bovine complex I. Other lanes are MEFs of fibroblasts from two controls (C) and two 3,460 subjects (IV1 and IV2.).
Figure 21. Western blot using a polyclonal antibody against bovine complex I holoenzyme. M kDa = molecular weight marker positions; H=human heart mitochondria (control subject); B=bovine complex I. Other lanes are MEFs from fibroblasts of two controls (C) and two 3,460 subjects (IV\textsuperscript{1} and IV\textsuperscript{2}).

6.5.2 Mitochondrial translation in 3,460 LHON

*In vitro* mitochondrial translation studies, as described in Chapter 4.9, revealed no qualitative differences in mitochondrial protein labelling between 3,460 LHON subjects (IV\textsubscript{1}, IV\textsubscript{2}, and III\textsubscript{2}) and age-matched controls (Figures 22,23). Protein synthesis was not observed in the presence of (+CAP). The bands have been recognized on the basis of their relative mobilities (Beattie and Sen, 1987; Bodnar et al. 1995) as ND5 (apparent M\textsubscript{r} 51kDa), COX\textsubscript{I} (44kDa), ND4 (39kDa), cytochrome b (35kDa), ND1 and ND2 (30kDa and
33kDa), COXII and COXIII which co-migrate at Mr 18-20kDa, ATPase 6 (25kDa), ND3 (13.6kDa), ATPase 8 (8kDa), and ND4L (10.5kDa).

Figure 22. $^{35}$S labelled mitochondrially synthesized proteins from fibroblasts of one control (C) and two LHON subjects (III2 and IV2), separated on a 16.5% SDS-PAGE containing 6M urea. M kDa = molecular weight marker positions. The lane between subject IV2 and C contained sample buffer only.
Figure 23. $^{35}$S labelled mitochondrially synthesized proteins from fibroblasts of one control (C) and three 3,460 LHON subjects (IV$_1$, III$_2$, and IV$_2$), separated on a 16.5% SDS-PAGE containing 6M urea. Products from the control in the presence of chloramphenicol are also shown (+CAP). M kDa = molecular weight marker positions.
6.6 Discussion:

Functional characterization of complex I in 3,460 LHON fibroblasts

The evidence supporting a complex I electron transfer defect in association with the 3,460b LHON mutation is overwhelming, as was discussed in Chapter 5.5. Electron transfer through complex I is linked to vectorial proton translocation across the inner mitochondrial membrane. The proton motive force generated is in turn used to synthesize ATP (Walker, 1992). Given this, it is difficult to explain the failure to show reduced ATP synthesis with complex I linked substrates in 3,460 LHON fibroblasts (Chapter 6.2). Before discussing the implications of such a result, an explanation of how normal ATP synthesis might be observed in the context of a apparent MRC electron transfer defect is required. This will be the subject of the next section.

6.6.1 Normal ATP synthesis associated with a severe MRC electron transfer defect: Hypotheses

6.6.1.1 Sensitivity of the ATP synthesis assay

The first possibility to consider is that the analysis of ATP synthesis might lack sufficient sensitivity to detect any decrease in ATP synthesis. This method has previously demonstrated the relationship between MRC enzyme function and ATP synthesis: Wanders et al. (1993) studied a single patient with a 75% COX defect in whom ATP synthesis with all substrates was significantly (78 - 94%) reduced, as expected given that electrons transfer through complex IV is a final common pathway. Robinson et al. (1986) using similar methodology assessed ATP synthesis in 3 infants with lactic acidosis and MRC defects. Two had isolated defects of rotenone sensitive NADH:cytochrome c reductase on spectrophotometric assays with activities 80% lower than those of controls. ATP synthesis with complex I linked substrates in these two patients was also almost 80% lower than in controls. Thus similar methods have shown
a good correlation between MRC function and ATP synthesis, including at the level of complex I.

To determine the sensitivity of the ATP synthesis assay, fibroblasts from patients with other mitochondrial diseases, and known MRC defects, would ideally have been studied in parallel to the LHON patients and controls. Unfortunately a suitable fibroblast line was not available. However it was possible to create a positive control by including rotenone in the incubations at concentrations known to inhibit complex I in control subjects, thus mimicking an electron transfer defect. The results in section 6.1 demonstrated that rotenone concentrations known to induce a 60% inhibition of NADH:CoQ₁ reductase activity in control fibroblasts, also produced a comparable 60% inhibition of ATP synthesis in the presence of complex I linked substrates. Together with the studies by Robinson et al. discussed above, the ATP assay thus appears able to detect a decrease in ATP synthesis consequent upon complex I dysfunction in cultured fibroblasts. Insufficient sensitivity of the ATP assay is not the explanation for normal ATP synthesis observed in 3,460 LHON fibroblasts.

6.6.1.2 Assessment of the complex I defect in 3,460 LHON

Given that ATP synthesis is normal in 3,460 LHON fibroblasts is it possible that complex I activity in these cells is in fact normal?

The spectrophotometric assay employed in this work has been widely used in this laboratory to demonstrate MRC defects in other mitochondrial diseases (Bodnar et al. 1993), in Parkinson's disease (Krige et al. 1992) and in studies of ageing (Cooper et al. 1992a). Others have agreed that the electron acceptor CoQ₁ is the best of the available ubiquinone analogues, with linear kinetics, relative independence to the amount of phospholipid present, and giving the highest activities (Estornell et al. 1993; Genova et al. 1995). This latter study none-the-less, did call into question the validity of NADH:ubiquinone reductase assays. Studying MRC function in ageing rats Genova et al. (1995) found that direct spectrophotometric quantification of rotenone sensitive
NADH:DB (decyl-ubiquinone) reductase activity significantly underestimated the complex I activity relative to the rate of total NADH oxidation, and the rate of ubiquinol oxidation. The authors point out that if validated this has implications for much of the work on diseases such as LHON, specifically quoting the data from 11,778 LHON studies where little/no defect in NADH:CoQ$_1$ reductase activity has been shown - suggesting that a defect may have been underestimated in the same way. This might explain why polarography and MRS detect defects where electron transfer appears normal as discussed in 5.6.1. However it is not possible to use the same hypothesis to account for the data in 3,460 LHON - if anything the NADH:CoQ$_1$ reductase defect might then be more severe than has been demonstrated.

The complex I assays used in the studies previously discussed rely on the specific inhibitor rotenone to differentiate mitochondrial complex I activity from that of other NADH:CoQ reductases. One possibility is that rotenone binding in 3,460 LHON fibroblasts is affected by the mutation. This has already been proposed in 11,778 LHON (Esposti et al. 1994). Rotenone inhibition of complex I probably involves dual binding sites, one hydrophilic and one within the lipid membrane, both of which must be occupied for maximum inhibition, possibly acting through a conformational change as inhibition is non-competitive with respect to ubiquinone (Singer and Ramsay, 1994). Photoaffinity studies show rotenone binds primarily to a 33kDa subunit which is probably ND1 (Earley et al. 1987). The 3,460 mutation substitutes an arginine for threonine in ND1, and could affect such a binding site. If rotenone binding in 3,460 LHON was significantly reduced compared to controls this would lead to an apparent defect in rotenone sensitive NADH:CoQ$_1$ reductase activity, where \textit{in vivo} electron transfer might be normal.

Consequently this question was directly addressed by performing rotenone sensitivity curves on MEFs from control and 3,460 fibroblasts. Rotenone sensitivity was altered in 3,460 LHON subjects (Chapter 6.2), but this was only apparent at very low rotenone concentrations (< 100nM). Standard
complex I studies, including those presented in Chapter 5, use 10μM rotenone to detect rotenone sensitive activity, 100 times greater than the highest level at which differences in rotenone sensitivity were apparent. Furthermore if abnormal rotenone binding accounted for the apparent defect in rotenone sensitive NADH:CoQ\textsubscript{1} reductase in 3,460 LHON then rotenone insensitive NADH:CoQ\textsubscript{1} reductase activity should be correspondingly increased in 3,460 LHON. The results presented in Chapter 5.4 show this not to be the case. Thus although the 3,460 mutation affects rotenone sensitivity, this effect is insufficient to account for the defect in rotenone sensitive NADH:CoQ\textsubscript{1} reductase activity demonstrated.

Finally using a different complex I inhibitor, DPI, the enzyme defect is still apparent. DPI binding has different characteristics to that of rotenone, and is thought to bind at a separate site on the 23.5kDa subunit (Ragan and Bloxham, 1977). Consequently the failure of DPI to significantly increase the inhibition of the NADH:CoQ\textsubscript{1} reductase activity in 3,460 LHON indicates that the decrease in complex I activity is not an artifact of rotenone binding. Although rotenone binding itself influences that of DPI, implying there is some relationship between DPI and the ND1 subunit, it seems unlikely that the 3,460bp mutation would also affect DPI binding to such an extent as to create the 85% defect in DPI sensitive NADH:CoQ\textsubscript{1} reductase seen.

Thus these data support a genuine defect in electron transfer from NADH to CoQ\textsubscript{1} in complex I from cells with the 3,460 LHON mutation.

6.6.1.3 NADH:CoQ\textsubscript{1} reductase kinetics

The complex I assay employed in these studies is carried out under \(V_{\text{max}}\) conditions, with the substrate (NADH) present in excess. This is a common approach to enzyme studies, and standardizes the conditions when material is limited. However the conditions may differ from those in vivo, and it is possible that at much lower concentrations of NADH the activity of mitochondrial NADH:CoQ\textsubscript{1} reductase in 3,460 cells would be comparable to
that of controls. To address this possibility the kinetics of NADH:CoQ₁ reductase were studied. However insufficient material was available for multiple data points to be obtained, especially at lower concentrations of NADH where with the material available the complex I activity was too low to be easily measured. Therefore although values for \(K_m\) were calculated from the data obtained, they must be interpreted with caution. From the data available, the \(K_m\) in the 3,460 subjects fell within the range of values obtained from the controls, and has not demonstrated any significant difference as a result of the 3,460 mutation. Previous kinetic studies of complex I in LHON (Majander et al. 1991) have also not documented \(K_m\) for NADH of rotenone sensitive NADH:CoQ₁ reductase in 3,460 subjects. Although the \(V_{\text{max}}\) of NADH:CoQ₁ reductase in 3,460 cells is clearly lower than in controls, if the electron turnover of complex I was near normal in LHON subjects at physiological concentrations of NADH, the finding of normal ATP synthesis in 3,460 LHON could be explained. This has not been excluded by the data presented, given the limitations discussed above, and thus remains a possibility in the \textit{in vivo} situation. Further kinetic studies in 3,460 LHON are required where a more pure mitochondrial preparation might be more suitable for this type of study.

6.6.1.4 Threshold effects.

Poor correlation between levels of electron flux through individual enzymes, and functional assessments of the intact MRC have been previously reported: Comparison of oxygen consumption with complex IV linked substrates, and spectrophotometric measurement of COX in rat muscle mitochondria were performed by Letellier \textit{et al.} in 1994. This revealed that increasing inhibition of COX with cyanide had little effect on oxygen consumption until high levels of COX inhibition were achieved. Even at 75% inhibition of the enzyme, the flux through the intact MRC was only 20% reduced, though beyond this the respiratory flux fell sharply - demonstrating a threshold effect. Similar results have been reported in rat liver mitochondria.
using myxothiazol to inhibit complex III (Taylor et al. 1994): only at > 45% inhibition of the enzyme activity was oxygen consumption also affected. Studies on thresholds with respect to complex I have also been reported: Letellier et al. in an earlier study (1993) compared rotenone inhibition of oxygen consumption with pyruvate and malate by rat muscle mitochondria, with that of NADH:CoQ₁ reductase activity. Some fall in respiration was seen with even low concentrations of rotenone, but >20% inhibition of oxygen consumption only occurred when complex I activity more than 70% inhibited. More recently Davey and Clark (1996) looked at rotenone inhibition of NADH:CoQ₁ reductase activity, oxygen consumption and ATP synthesis with complex I linked substrates in non-synaptic rat brain mitochondria, and demonstrated that only when complex I was greater than 72% inhibited was any significant effect on MRC flux seen. These studies support the existence of a similar threshold effect in rat complex I, although Letellier et al. (1993) were more cautious in their interpretation of the complex I results as the metabolic control model applied (Gellerich et al. 1990) was not a good fit for rotenone inhibition curves. Polarographic data has not been reported from 3,460 LHON subjects, but ATP synthesis reflects the overall function of the MRC and oxidative phosphorylation system. Thus perhaps the threshold of complex I activity above which electron flux through the intact MRC is affected is higher than the ~60% enzyme defect in seen in the presence of the 3,460bp mutation. However the direct relationship between rotenone induced complex I inhibition and ATP synthesis in control fibroblasts suggests this is not the case. A threshold effect does not explain normal ATP synthesis in 3,460 LHON fibroblasts.

A tissue specific threshold in the optic pathways cannot be excluded, and inter-tissue variability in such thresholds has been reported: the complex III inhibitor studies discussed above which demonstrated a 45% threshold in rat liver, found in muscle that only 5% inhibition of enzyme resulted in a fall in respiratory flux (Taylor et al. 1994). It was considered by the authors that liver mitochondria might be working well below their maximal capacity, and thus be
able to lose considerable activity before oxidative phosphorylation was significantly affected. Applying similar reasoning to these complex I results, one would expect fibroblasts to have lower metabolic requirements than nervous tissue, and if anything to tolerate considerable enzyme inhibition before ATP synthesis was affected. The rotenone inhibition results in fibroblasts do not support this, but in contrast to other studies on threshold effects the conditions used for ATP synthesis and enzyme studies are very different. The previous studies have all used mitochondrial preparations, whereas in this work permeabilized whole cells were used for ATP synthesis assays, and MEFs for spectrophotometric assays. Absolute concentrations of rotenone in the reaction mixtures were equal, but differed considerably in terms of rotenone:mitochondrial protein ratios. Each ATP incubation contained 1-2mg of cellular protein, with an unknown mitochondrial protein content, whereas enzyme assays used 40 - 80μg of protein of a MEF. Thus it could be argued that direct comparison of ATP results with those of complex I enzyme activities is inappropriate. However fibroblasts ATP synthesis with complex I linked substrates was clearly inhibited by even the lowest concentrations of rotenone, and no threshold effect demonstrated. Other explanations for normal ATP synthesis in 3,460 fibroblasts expressing a severe complex I defect must be considered.

6.6.2 Electron transfer paths through complex I

Little is known about the pathways of electron transfer and mechanisms relating to proton translocation in complex I. Detailed studies of electron transfer have been hampered by the lack of specific inhibitors for different sites in complex I. Most to date relate to the NADH or quinone binding sites. The most recent studies show NADH probably binds to the 51kD subunit in the flavoprotein fraction, with FMN as the immediate oxidant having a reduction potential intermediate between that of NADH and the first Fe-S cluster (reviewed by Walker, 1992). There are at least 5 Fe-S clusters in bovine
complex I and electrons would finally pass from the highest potential cluster to the acceptor ubiquinone, possibly via an intermediate ubisemiquinone radical (Vinogradov et al. 1995). Being lipophilic, ubiquinone is likely to be present within the mitochondrial membrane, and may bind to, or near to ND1 (Singer and Ramsay, 1994). The mechanisms of proton translocation in complex I are also unknown and may involve only energy transduction, or a redox-driven conformational change (Singer and Ramsay, 1994). Known inhibitors of proton translocation in other enzymes such as dicyclohexylcarbodiimide (DCCD) inhibit electron transfer and proton translocation in parallel in complex I, and bind to a protein whose sequence corresponds to that of ND1 (Yagi and Hatefi, 1988). This suggests ND1 might be important in both electron transfer and proton translocation, but this has yet to be confirmed.

It has been proposed that there are at least two separate electron transfer paths through complex I: one supporting the oxidation of NADH at pH 8.0, and another the oxidation of NADPH at pH 6.5 (van Belzen and Albracht, 1989). Rotenone inhibits both, and probably acting at a final common pathway between the final Fe-S cluster and ubiquinone (Singer and Ramsay, 1994). If the kinetic differences in complex I in 3,460 fibroblasts, or the existence of a threshold effect, do not account for normal ATP synthesis in 3,460 LHON fibroblasts, the model of complex I described above cannot account for normal ATP synthesis where a severe defect in rotenone sensitive electron transfer has been demonstrated. Rotenone sensitive NADH:CoQ₁ reductase activity is not a comprehensive assay of complex I function. It is possible that additional electron transfer pathways exist, not detected by this approach. An additional, rotenone insensitive electron transfer pathway able to utilize complex I linked substrates might be supporting ATP synthesis in 3,460 LHON fibroblasts. The rotenone sensitive electron transfer, observed to be decreased in 3,460 LHON, would not be linked to proton translocation in such a model. Such additional pathways have not been directly demonstrated, but the most recent inhibitor studies (Anderson and Trgovcich-Zacok, 1995) do support the existence of
additional electron transfer routes through complex I: Anderson and Trgovcich-Zacok screened 13 different complex I inhibitors with different substrates at varying pH values, and identified compounds in four different groups. A two-pathway electron transfer model would predict at most three categories of complex I inhibitor - one inhibiting each pathway independently, and one inhibiting both. The authors proposed that at least 3 electron transfer paths, including one from NADH at pH 6.5 not present in earlier models, must exist. This model would predict at least 7 inhibitor categories based on differential effects, of which they were able to characterize four. Most currently identified inhibitors, including rotenone, inhibit all pathways. Although this data cannot directly account for the findings presented in this Chapter, it supports the possibility of additional electron transfer pathways perhaps able to functionally compensate where traditional routes are affected by mutations such as that in 3,460 LHON.

The explanation for normal ATP synthesis in 3,460 LHON fibroblasts remains unclear. A threshold effect in the relationship between inhibition of complex I and inhibition of ATP synthesis has not been demonstrated in this study. The existence of additional electron transfer pathways through complex I, or that the effect of the mutation on complex I kinetics in vivo is negligible remain possibilities.

6.6.3 Protein studies in 3,460 LHON

Detailed studies on the protein structure of complex I in LHON have not been reported. The results of Western blot analysis and in vitro translation studies in 3,460 LHON suggest that the synthesis of mitochondrially encoded complex I subunits, and assembly of the final holoenzyme is relatively normal. The ND1 subunit in 3,460 LHON migrated at the same distance as that of controls, and no additional bands were seen. The arginine for threonine substitution resulting from the 3,460bp mutation does not seem to affect the structural composition and assembly of complex I in fibroblasts. Western blots
using monoclonal antibodies against human complex I subunits would be expected to be much more specific and may be more informative, but such antibodies were not available to us. In addition antibodies directed against the mitochondrially encoded subunits are hard to generate as these subunits, being hydrophobic and less antigenic, are difficult to purify. Similar methodology has identified deficiencies in complex I subunits in other mitochondrial disease where complex I is functionally abnormal (Schapira et al. 1990b; Slipetz et al. 1991), and were the composition or assembly of complex I in 3,460 fibroblasts significantly deranged. Thus significant abnormalities in this study should have been apparent, although the mitochondrial preparations used in this study were less pure than in the work by Schapira et al. (1990b) and Slipetz et al. (1991), so the signal achieved was fairly weak and hence harder to interpret.

These data do not exclude the possibility that the total levels of complex I protein in 3,460 fibroblasts is different from that of controls, as no strictly quantitative methodology was applied. However as no consistent differences in band intensity were seen between control and 3,460 subjects when equal total protein loads/total radioactive counts were studied, it is unlikely that there is any significant difference in absolute complex I levels in 3,460 LHON fibroblasts compared to those of controls. By implication, an excess of abnormally functioning complex I does not account for the normal ATP synthesis seen in 3,460 fibroblasts.

6.6.4 Functional consequences of the 3,460 mutation in the clinical context

The pathogenesis of mitochondrial diseases has generally been considered to involve decreased ATP synthesis consequent upon MRC enzyme defects, resulting in a reduced capacity of the cell to carry out ATP-dependant physiological functions, and in at least some instances cell death. LHON has to date been included in this remit, yet clinically LHON is in most cases a largely tissue specific disease. This is not reflected in the tissue distribution of either the mtDNA mutation or the biochemical defect. Furthermore if the functional
consequences of the 3,460 LHON mutation do not include a reduction of the cell's ability to produce ATP, the pathogenetic mechanisms must be reconsidered.

No clinical problems with fibroblast function are apparent in LHON, though the needs of fibroblasts for ATP might be so low that even if a significant defect was present the physiological consequences would be negligible. Studies of physiological or mitochondrial function in tissues highly dependent on oxidative metabolism such as muscle have not been reported in 3,460 LHON, but muscle disease is not a clinical/symptomatic feature of LHON so we can infer that function in physiological terms is also not severely deranged in muscle. A poor correlation between biochemical defects and clinical expression is well recognized in mitochondrial diseases as a whole, but the homoplasmy and degree of complex I inhibition in 3,460 LHON is difficult to reconcile with the clinical presentation. From a clinical point of view normal ATP synthesis, not only in 3,460 LHON fibroblasts, but also in LHON muscle, brain, liver, and heart, would be entirely consistent with the rarity of symptoms outside the optic pathways. This leaves us with the hypotheses that either the biochemical consequences of the 3,460 mutation, and indeed of other LHON mutations, are either somehow of much greater functional significance in the clinically affected optic pathways, or that biochemical dysfunction in LHON is not of pathogenetic significance at all. This will be considered further in the final LHON discussion (Chapter, 8).
Chapter 7: Nuclear genomic complementation in 3,460 LHON

Introduction:

The idea that factors other than mtDNA mutations must be involved in the clinical expression of LHON is not novel, as was introduced in Chapter 2.7. A male homoplasmic for a primary mutation has only a 50% chance of developing symptoms, and this falls to 15% for females (Riordan-Eva and Harding, 1995; Oostra et al. 1994a). The male excess of clinically affected individuals is estimated at 4.3:1 based on eight 3,460 pedigrees in a recent study (Harding et al. 1995), and neither mtDNA nor functional mitochondrial analysis has yet been able to distinguish clinically affected from unaffected carriers of the mutation. This combination of factors has lead to the search for other potential influences on clinical expression, in particular potential X-linked susceptibility genes. Pedigree analysis of 31 families by Bu and Rotter (1991) and of 85 genetically confirmed families by Harding et al. (1995) support a two-hit model involving both a pathogenic mtDNA mutation and an X-chromosome susceptibility gene as best fitting the pattern of clinical expression seen. Females would become symptomatic if homozygous for the X-linked gene, or through disadvantaged X-inactivation in heterozygous germline cells. Initial reports suggesting linkage to the DXS7 locus (Vilkki et al. 1991) have since not been confirmed (Sweeney et al. 1992; Juvonen et al. 1993), although none set penetrance at values supported by the pedigree studies, and areas of the X-chromosome have not yet been excluded in linkage analysis. An alternative approach to investigate the possible interaction between nuclear and mitochondrial genomes in the pathogenesis of LHON is to examine the influence of a control (non-LHON) nuclear environment on the biochemical expression a LHON mutation in tissue culture. LHON cells expressing a clear biochemical defect are best suited for such experiments. Fibroblasts from subjects with the 3,460 LHON mutation have been shown to express a 60% defect in complex I activity. If this defect is solely consequent upon the mitochondrial mutation this defect should persist in a new non-LHON nuclear
environment. This hypothesis can be tested by fusing enucleated LHON fibroblasts with mtDNA-less \((\rho^0)\) cells, followed by genetic and biochemical analysis of the resultant clonal cybrids containing 3,460bp LHON mtDNA but a different nuclear genome. For these studies a \(\rho^0\) cell line derived from a human lung carcinoma (A549) was used, with methodology as described in chapters 4.2.7 and 4.2.8.

7.1 Characterization of the A549\(\rho^0\) cell line

7.1.1 Growth requirements of the A549\(\rho^0\) cells

Triplicate growth curves performed on the A549\(\rho^0\) cells confirmed their dependence on pyruvate and uridine in the medium. In selection medium (lacking pyruvate and uridine) A549\(\rho^0\) cells not only failed to grow but started to die within a few days (Figure 24).

![Figure 24](image)

Figure 24. Growth curves of A549\(\rho^0\) cells in growth (supplemented) medium and selection medium (lacking pyruvate and medium). Points shown are mean +/- SD. Where not indicated SD values were insufficiently large to plot.
7.1.2 MRC function in the A549\(\rho^0\) cells

PCR using the 3,460bp LHON primers failed to produce a product, indicating the absence of mtDNA in these cells (Figure 25). Standard spectrophotometric enzyme assays were carried out on MEFs prepared from cultured A549\(\rho^0\) cells and compared to those of the parental (+mtDNA) A549 cell lines. The CS in the A549\(\rho^0\) cells was significantly lower than in the parental cell line (Table 13). No complex II/III or IV activity was detected. Total NADH:CoQ\(_1\) reductase activity in A549\(\rho^0\) cells was higher than in the parental A549 cells, but this difference was not significant and rotenone failed to inhibit this rate confirming a lack of mitochondrial complex I activity (Table 13). A small reduction in NADH:CoQ\(_1\) reductase activity (mean sensitive rate 3.82 +/- 4.14 nmols/min/mg protein) was seen with the addition of 10mM (f.c.) rotenone in 10μl ethanol, but this was not significantly different from the change seen if ethanol alone was added. No rate specifically inhibitable by rotenone was demonstrable (mean inhibited rate 4.53 +/- 4.79 nmols/min/mg protein). These cells were thus suitable hosts in which to study the influence of a different nuclear environment on the expression of the 3,460bp mtDNA mutation.
Figure 25. PCR products using primers for a 630bp fragment of mtDNA from cell lysates of control-A549p0 cybrids (Cont1-p0) and A549p0 cells.
Table 13. MRC enzyme activities in MEFs from A549 cell lines.

A: specific activities

<table>
<thead>
<tr>
<th></th>
<th>CS</th>
<th>NADH:CoQ₁ R rot. insens.</th>
<th>NADH:CoQ₁ R rot. sens.</th>
<th>SCcR</th>
<th>COX</th>
</tr>
</thead>
<tbody>
<tr>
<td>A549</td>
<td>170.7 +/- 41.5</td>
<td>58.2 +/- 24.4</td>
<td>44.0 +/- 17.5</td>
<td>70.8 +/- 18.4</td>
<td>3.5 +/- 0.4</td>
</tr>
<tr>
<td>A549ρ₀&lt;sup&gt;0&lt;/sup&gt;</td>
<td>99.7 +/- 8.5*</td>
<td>74.7 +/- 24.4</td>
<td>ND**</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

B: Citrate synthase ratios

<table>
<thead>
<tr>
<th></th>
<th>NADH:CoQ₁ R rot. insens.</th>
<th>NADH:CoQ₁ R rot. sens.</th>
<th>SCcR</th>
<th>COX</th>
</tr>
</thead>
<tbody>
<tr>
<td>A549</td>
<td>0.377 +/- 0.213</td>
<td>0.26</td>
<td>0.420</td>
<td>0.022</td>
</tr>
<tr>
<td></td>
<td>+/- 0.116</td>
<td>+/- 0.076</td>
<td>+/- 0.004</td>
<td></td>
</tr>
<tr>
<td>A549ρ₀&lt;sup&gt;0&lt;/sup&gt;</td>
<td>0.7653 +/- 3.018</td>
<td>ND**</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

A: specific activities are expressed as nmols/min/mg protein except for COX activity which is expressed as the first order rate constant k/min/mg protein. B: data from A expressed as a ratio with CS activity. Values are means +/- SD of three preparations. ND = not detected; * p < 0.05. ** see text for details.
7.2 Growth characteristics of fibroblasts harbouring the 3,460bp LHON mutation

Control and mutant fibroblasts were not distinguishable in culture on the basis of the light microscopy appearance of the cells, replicative capacity or growth rates. Growth curves performed in triplicate on 3,460bp LHON fibroblasts and controls demonstrated that although both grew slower in selection medium lacking pyruvate and uridine, there was no preferential selection against cells homoplasmic for the 3,460bp mutation, known to express a complex I defect. (Figure 26).

Figure 26. Growth curves of control (plain symbols) and 3,460 (filled symbols) LHON fibroblasts (patient IV1) in growth (supplemented) medium and selection medium (lacking pyruvate and uridine). Points represent means +/- standard deviations. Where not indicated SD values were insufficiently large to plot.
7.3 Enucleation conditions and efficacy

The efficiency of enucleation was assessed by visual estimation of the number of cells retaining nuclei when stained with Mayer's haematoxylin as described in Chapter 4.2.7. Cytoplasts were only used for fusion experiments if a trial coverslip enucleated under identical conditions resulted in >90% enucleation. Examples of successful and poor enucleations are shown in Figure 27. The conditions used for each of the cell lines fused are listed in Table 14. Several trial runs were required for each cell line as some variation in optimum conditions between cells and on different days was seen.

Table 14. Conditions yielding >90% enucleation for each of the cell lines fused.

<table>
<thead>
<tr>
<th>Fibroblasts</th>
<th>temp. (°C)</th>
<th>time (mins.)</th>
<th>cytochalasin b μg / ml</th>
<th>g_max</th>
<th>enucleation %</th>
<th>cell loss %</th>
</tr>
</thead>
<tbody>
<tr>
<td>22y control</td>
<td>35</td>
<td>20</td>
<td>10</td>
<td>25,000</td>
<td>90</td>
<td>60</td>
</tr>
<tr>
<td>46y control</td>
<td>35</td>
<td>22</td>
<td>10</td>
<td>30,000</td>
<td>95</td>
<td>60</td>
</tr>
<tr>
<td>IV1</td>
<td>35</td>
<td>19</td>
<td>10</td>
<td>25,000</td>
<td>90</td>
<td>50</td>
</tr>
<tr>
<td>III2 Clone2</td>
<td>35</td>
<td>19</td>
<td>10</td>
<td>25,000</td>
<td>90</td>
<td>40</td>
</tr>
</tbody>
</table>
Figure 27. Poor (A) and >90% (B) enucleation of fibroblasts. Cytoplasts and cells have been fixed in ethanol:acetic acid and stained with Mayer's haematoxylin after centrifugation in cytochalasin b. Nuclei show as dark purple spots, with cytoplasm staining only faintly. The cytoplasts are abnormally stretched and have a "stringy" conformation as a result of the high centrifugal force. This resolves spontaneously after 2 - 3 hours recovery in medium.
7.4 DNA analysis of A549ρ0- fibroblast cybrids.

7.4.1 mtDNA analysis

Two control fibroblast lines (males aged 22 and 46) were enucleated and the resultant cytoplasts fused with A549ρ0 cells. This yielded 13 and 9 control-A549ρ0 cybrid clones from each of the controls respectively. Restriction enzyme digestion of PCR products from these cybrids confirmed the presence of mtDNA lacking the 3,460bp mutation (Figure 28). The undigested PCR product generated with these primers (Chapter 7.4) is 630bp. Wild-type mtDNA digests into fragments of 100bp and 530bp, whereas the 3,460bp mutation results in a loss of this restriction site. Fibroblasts from IV1 and a clonal line from III2 (clone 2), each containing 100% mutant 3,460 LHON mtDNA were also enucleated and fused with A549ρ0 cells, yielding 6 and 7 3460-A549ρ0 cybrid clones respectively. MtDNA analysis confirmed that these contained only mtDNA with the 3,460 LHON mutation, as the 630bp fragment failed to digest in all fusion clones indicating a loss of the Hin1 1 restriction site (Figures 29,30).

Homoplasmy for the 3,460bp mutation in 3,460-A549ρ0 fusion clones persisted in culture at higher passage numbers (Figure 31). Two sample control-A549ρ0 cybrids have only wild-type mtDNA, whereas in all the 3,460-A549ρ0 cybrids the loss of restriction site representing mutant mtDNA has persisted. PCR products from the original A549ρ0 are also shown here, with no visible PCR products.
Figure 28. *Hind*I 1 restricted PCR products from control-A549ρ0 cybrid clones (labelled 1-9), separated on a 1.2% agarose gel. Cybrids were generated by fusing enucleated fibroblasts from a 46y control with A549ρ0 cells. U represents the unrestricted PCR product.

Figure 29. *Hind*I 1 restricted PCR products from control-A549ρ0 (C) and III2(clone 2)-A549ρ0 cybrid clones (labelled 1-6), separated on a 1.2% agarose gel.
Figures 30. *Hinl* 1 restricted PCR products from control-A549ρ₀ (C) and IV₁-A549ρ₀ cybrid clones (labelled 1-7), separated on a 1.2% agarose gel.

Figure 31. *Hinl* 1 restricted PCR products from high passage number A549ρ₀ cells (ρ₀), and 3,460-A549ρ₀ (IV₁- and III₂-ρ₀) and control-A549ρ₀ (Cont₁-ρ₀) cybrid clones separated on a 1.2% agarose gel. p denotes the passage number, and cell lines are as shown.
7.4.2 Nuclear DNA analysis

Nuclear genomic analysis of IV1- A549Δ cell hybrid clones numbers 3 and 5, using multi-loci and single-loci probes was used to assess the origin of their nuclear material. The multi-loci fingerprint for fibroblasts from the LHON patient IV1 demonstrated 29% homology with the A549 cell line which is within acceptable limits (Figure 32). Cybrid cell lines IV1- A549Δ clones 3 and 5 had fingerprint patterns indistinguishable from those of the parental A549 cells, and possessed no bands unique to fibroblasts from IV1. This is consistent with the nuclear material in the IV1- A549Δ cybrid clones being that of the A549 cells. No evidence of nuclear material from IV1 was demonstrated. The results using single-loci probes were also consistent with this conclusion (data not shown).

Figure 32. Genetic fingerprints using the multiloci probes (MLP) 33.15 and 33.16. M = DNA marker; Sample DNA from: 3,460-Cybrid clones 3 and 5 (IV1-A549Δ); patient IV1 fibroblasts (IV1); parental A549 cells (A549).
7.5 MRC function in 3,460-A549ρ0 fusion cybrids

Eight control-A549ρ0 cybrid clones (4 from each of the 2 controls fused) and 7 of the 3,460- A549ρ0 cybrid clones (3 from III2- A549ρ0 and 4 from IV1- A549ρ0) were randomly selected for functional analysis. MRC enzyme assays were performed using identical methods to those used for the original cell lines. The mean activities of citrate synthase, complex I, complexes II/III and IV in the 3,460-A549ρ0 cybrid clones as a group were not significantly different from those of control-A549ρ0 cybrid clones (Table 15; Figure 33). When individual clones were compared two of the IV1- A549ρ0 lines (clones 3 and 6 in Table 15 and Figure 33) had complex I:CS ratios which fell outside the range of control values, and were consistently much lower than in the other clones analysed. When data from these two cybrid clones were considered separately a significant complex I defect was seen when compared to the mean values from control cybrids (63% decreased; p = 0.044), although when included in the group of seven 3,460-A549ρ0 cybrid clones no complex I defect was apparent in association with the 3,460bp mutation. Likewise when the other five 3,460-A549ρ0 cybrid clones were grouped and compared to controls, there was no significant difference in complex I activity.
Table 15. MRC enzyme activities in MEFs from 3,460-A549ρ0 cybrids and control-A549ρ0 cybrids.

A

<table>
<thead>
<tr>
<th>Cells</th>
<th>Citrate synthase</th>
<th>NADH:CoQ₁ R rot.insens.</th>
<th>NADH:CoQ₁ R rot. sens.</th>
<th>SCCr</th>
<th>COX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control-ρ₀ (n=8)</td>
<td>125.3 +/- 17.1</td>
<td>79.7 +/- 24.0</td>
<td>40.7 +/- 11.8</td>
<td>68.9 +/- 9.0</td>
<td>3.38 +/- 0.74</td>
</tr>
<tr>
<td>3,460-ρ₀ (n=7)</td>
<td>135.08 +/- 22.64</td>
<td>90.19 +/- 25.74</td>
<td>42.22 +/- 16.75</td>
<td>69.84 +/- 20.23</td>
<td>2.854 +/- 0.519</td>
</tr>
<tr>
<td>#IV₁-ρ₀ clone 3; clone 6</td>
<td>109.1; 173.1</td>
<td>83.7; 47.0</td>
<td>18.6; 23.8*</td>
<td>43.9; 102.5</td>
<td>2.41; 3.91</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>Cells</th>
<th>NADH:CoQ₁ R rot.insens. (X10)</th>
<th>NADH:CoQ₁ R rot. sens. (X10)</th>
<th>SCCr (X10)</th>
<th>COX (X100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control-ρ₀ (n=8)</td>
<td>6.78 +/- 2.63</td>
<td>3.39 +/- 1.30</td>
<td>5.59 +/- 0.61</td>
<td>2.71 +/- 0.31</td>
</tr>
<tr>
<td>3,460-ρ₀ (n=7)</td>
<td>6.72 +/- 1.74</td>
<td>3.17 +/- 1.32</td>
<td>5.21 +/- 0.98</td>
<td>2.27 +/- 0.20</td>
</tr>
<tr>
<td>#IV₁-ρ₀ clone 3; clone 6</td>
<td>4.84; 4.37</td>
<td>1.39; 1.71*</td>
<td>6.05; 4.05</td>
<td>2.37; 2.21</td>
</tr>
</tbody>
</table>

A: results are expressed as nmols/min/mg protein except for COX which is expressed as k/min/mg protein. B: data from A expressed as a ratio with CS activity. Values are means +/- SD. n represents the number of cybrid clones analysed. # The mean of triplicate preparations of two of the clonal lines have been listed separately as discussed in the text. * p<0.05 when the values from these two clones are combined and compared to the control group using the Mann Whitney U-test.
7.6 Discussion: Nuclear genomic complementation in 3,460 LHON

7.6.1 MRC function in A549 cells

The suitability of the A549ρ⁰ cells for this study depended on their having no demonstrable intrinsic MRC enzyme activity prior to the introduction of mitochondria derived from patients fibroblasts. That no complex I, II/III or IV activity was detected using standard assays confirmed that they were suitable hosts in this respect.

The lower CS seen in the A549ρ⁰ cells compared to the parental (+mtDNA) A549 cells may reflect either less CS activity/mitochondrion than in the parental cells, or less pure MEFs. Low CS activity has not been demonstrated in other studies using these cells (Bodnar et al. 1993) so protein impurity was considered a more likely explanation. In addition as the activities from 3,460-A549ρ⁰ cybrids were compared to those of control-A549ρ⁰ cybrids, and not the parental A549 line any alterations in CS that have occurred in the
A549ρ0 cells prior to fusion should be expressed in both the control and the 3,460 cybrids subsequently generated and not affect interpretation of the results.

The reasons for the higher total NADH:CoQ₁ reductase activity in the A549ρ0 cells compared to the parental A549 cells are not known. Although this difference was not statistically significant it was important to establish that complex I activities could be reliably interpreted in cybrids derived from the A549ρ0 cells. Certainly other NADH dehydrogenases are known to exist within cells, and only a fraction of the total enzyme activity is considered to represent mitochondrial complex I activity. It may be that in the absence of mitochondrial complex I activity other NADH dehydrogenases are induced, and measured by the NADH:CoQ₁ reductase assay. However it is only the rotenone sensitive NADH:CoQ₁ reductase rate that is of primary concern in this study, and no such specifically rotenone-sensitive activity was present.

7.6.2 Restoration of normal complex I activity to 3,460 cells by a new nuclear environment

This work suggests that the biochemical defect previously attributed to the 3,460 LHON mutation is corrected in the presence of a control nuclear environment in at least five of seven cell lines studied, despite persistence of the 3,460 mtDNA mutation. The A549-ρ0 cell line has been reported by others to preferentially propagate wild-type mtDNA (Dunbar et al. 1995) from heteroplasmic donors, but in this study persistence of the homoplasmic 3,460 mtDNA genotype in each of the cybrids studied was confirmed. However in order to interpret the data reliably a number of other factors require discussion.

7.6.2.1 Complex I variability in A549-ρ0 cybrids.

The variation in complex I activity in A549-ρ0 cybrids was greater than that observed in either the parental A549 cells or the donor fibroblast lines, either from controls or patients. In controls the Complex I:CS ratios between
individual cell lines varied by a maximum of 190\% (n = 9; range 0.125 to 0.288), whereas in control-A549pO cybrid cell lines the maximum variation was higher at 318\% (n = 8 cybrid clones from 2 patients; range 0.175 to 0.554). The reason for this increased spread of data is not clear but it makes confident interpretation of the results more difficult. It is something that has been observed by other groups (Chomyn et al. 1994), and attributed to variable nuclear backgrounds between the pO cells themselves. Transformed cells do have inherently unstable nuclei with frequent chromosomal aberrations observed, and the potentially mutagenic effect of several weeks growth in ethidium bromide may only add to this phenomenon. If this is the case, using A549- pO cells that have been recently cloned might overcome this difficulty.

Despite these observations the complex I:CS values in the 3,460 fibroblasts all fell outside the control range, whereas after fusion all but two of the 3,460-A549pO cybrid clones had activities within the range seen in control cybrids as shown in Figure 33. The complex I:CS ratios outside the control range (IV1A549- pO clones 3 and 6) fell only 20.5\% and 3.5\% below the lowest control value. The spread of complex I activities from the 3,460-A549pO cybrids contrasts with the situation in the 3,460 fibroblasts, from which the highest complex I:CS ratio was still 40\% lower than the lowest control value. Thus despite the increased spread of data the pattern has clearly changed, and in at least 5 of the 7 3,460-A549pO cybrid clones analysed complex I activity was within the normal range.

That two 3,460-A549pO lines fell outside the range of control values may reflect only the increased spread of data, and these may still be considered "corrected". Alternatively it is possible that there is a persistent complex I defect in these two cell lines, despite the presence of the A549- pO nucleus. There are two possible explanations for this. Firstly as the enucleation process is at best only 90\% efficient it is possible that some of the cybrids might represent fusion products of the A549pO cells and nucleated LHON fibroblasts. The selection process would not preclude survival of such a cell line. LHON nuclear material
might thus still be present in the uncorrected cybrids and would be expressed if determined by a dominant gene. However the genetic fingerprinting did not suggest the presence of any nuclear material from patient IV₁ in these fusions making this unlikely, although not impossible. Alternatively IV₁ might be heteroplasmic for an as yet unidentified additional mtDNA mutation, which is contributing to the biochemical defect. The mtDNA of IV₁ would be expected to be identical to that of III₂. However a clonal line from III₂ was used for fusion studies and might by chance not have possessed such a second mutation. Against this is the fact that none of the other primary (11,778,14,484) or secondary (including 4,160) mutations have been identified in this family. Any putative second mtDNA mutation responsible for the complex I defect identified in our cells would also have to have disappeared in the 0% 3,460bp clone to account for normal complex I activity in these cells. This seems improbable as only two of our fused cell lines failed to correct, implying that the 3,460bp and the putative second mtDNA mutation do not necessarily segregate together.

Insufficient data about the reasons for the increased spread of data in A549-ρ₀ cybrids, and for the higher complex I activities in general in these cells is available to account for this discrepancy. Analysis of more controls and patients in the future, using this and other non-LHON nuclear environments may allow a more definitive explanation.

7.6.2.2 MtDNA levels in A549ρ₀ cybrids

One possibility to account for the variation in complex I activity seen in A549ρ₀ cybrids is that MRC function is related to absolute mtDNA levels in these cells. Comparable variation was not seen for other MRC enzymes, but this might reflect the fact that fewer subunits in other enzymes are mitochondrially encoded. The factors that control mtDNA replication are poorly understood. Traditionally it is believed that in mature cells, such as fibroblasts, mtDNA is replicated once with each cell generation, thus maintaining a steady state (Clayton, 1992). It has been shown that in ρ₀ cells these normal
restraints on mtDNA replication are lacking: microinjection of a single mitochondrion into a \( \rho^0 \) cell results in normal levels of mtDNA within less than 35 generations, as assessed by dot-blot hybridization (King and Attardi, 1989). Thus clearly the rate of mtDNA replication has exceeded that of cell replication in order to achieve this. Presumably, as in many biological systems, a feedback loop exists controlling mtDNA replication in response to need. In a cell that has been artificially deprived of its own mtDNA, signals stimulating mtDNA replication might thus be very active, although inactivation of a negative feedback loop is equally possible. In either case it is possible that mtDNA levels in individual clonal cybrid lines, not subject to the normal controls, are unequal. It is well established in many mitochondrial diseases that the mtDNA genotype, and the proportion of mutant mtDNA present influences respiratory competence. In extreme cases we also know that absolute levels of mtDNA are important as in the mitochondrial depletion syndromes (Moraes et al. 1991).

One variable to be considered then is the absolute levels of mtDNA in these cybrid colonies, specifically at the passage number relating to biochemical results. If mtDNA levels differ significantly between individual cybrid cell lines, and this in turn directly affects respiratory chain function it could either mask or give differences unrelated to the nuclear environment. For instance in the 3,460-A549\( \rho^0 \) cybrids that appear to have corrected, this might merely reflect much higher levels of mtDNA, perhaps produced in response to the respiratory deficiency caused by the mutation. Such compensatory behaviour might not be possible in the original fibroblasts, due to the inherent regulatory mechanism previously proposed. Thus the "nuclear complementation" would in fact only reflect disruption of such mechanisms, and not the replacement of a specific LHON susceptibility gene.

However other than at very low levels of mtDNA as in depletion syndromes, there is little evidence that absolute levels of mtDNA contribute significantly to respiratory competence either in original cell lines or artificially generated cybrids. In the original paper by King and Attardi (1989), the range of
mtDNA levels seen in the cybrid lines did not differ significantly from each other, or from the parental lines. Similarly Chomyn et al. (1991) found similar levels of mtDNA in all cybrid clones, whether expressing mutant or wild-type mtDNA. The biochemical phenotype related only to the proportion of mutant, not to absolute mtDNA levels, and good correlation was seen in this respect. Admittedly both these papers used a different host cell to our own, specifically an osteosarcoma derived $\rho^0$ line, but the method of mtDNA depletion was the same and there is no reason to expect the A549-$\rho^0$ cells to differ in this respect. The same group in 1994 using platelets as mtDNA donor cells found extreme variability both in respiratory function and in mtDNA levels in the resultant cybrids, but these two variables did not correlate and it was concluded that other factors, in particular the nuclear background and not absolute mtDNA levels, were responsible for the variability in enzyme activities (Chomyn et al. 1994). A different nuclear background was implicated as contributing to MRC function as variation in respiratory function that was demonstrated between cybrid clones from an individual donor, was not maintained after a further mitochondrial transfer experiment, involving enucleation of the original cybrid clones and fusion of these cytoplasts with a second nuclear environment. Others have also documented "normal" amounts of mtDNA in $\rho^0$ cybrids, and no observable change in either absolute levels or % mutation 4 - 8 weeks after fusion (Mariotti et al. 1994).

Quantification of mtDNA levels in these 3,460-A549$\rho^0$ or control-A549$\rho^0$ cybrids was not performed, and this should be rectified in any future work. However there is no current evidence to suggest that the conclusions so far drawn should be reconsidered on this basis.

7.6.3 Other cybrid studies in LHON

A study on 11,778-$\rho^0$ cybrids (mtDNA originating from platelets with the 11,778bp LHON mutation) concluded that the respiratory phenotype of these cells was dependent on the mtDNA type, and not the nuclear environment
(Vergani et al. 1995). However data on the original non-fused LHON platelets was not presented in this paper. Other studies in 11,778 LHON have not detected a significant complex I defect in primary cell lines which have exhibited either normal electron transfer activity (Larsson et al. 1991; Majander et al. 1991), or only a mild defect (Smith et al. 1994). The 11,778-p0 cybrids generated by Vergani et al. (1995) from one subject exhibited a 40 - 50% defect in complex I activity (NADH:CoQ1 reductase) compared to control cybrids. The finding of such a severe defect in these cybrids, if it is not expressed in primary 11,778 cells, is hard to explain. In fact when data from this subject was combined with results from cybrids from a second 11,778 LHON subject, no significant difference in complex I electron transfer was present in 11,778 cybrids compared to the controls. Oxygen consumption in this study was significantly decreased in 11778-p0 cybrids compared to controls, and this has also been well documented in original 11,778 cell lines (Larsson et al. 1991; Majander et al. 1991), suggesting that this feature is indeed a attributable to the mutant mtDNA and not the nuclear environment. However complex I linked substrates were not specifically used, and one of the 2 patients studied was atypical for LHON in that he also had a lactic acidaemia, a spastic paraparesis, myopathic changes on muscle biopsy, and widespread MRI changes compatible with demyelination. Only the results from this patient's cybrid clones fall clearly outside the control range. He was known also to possess the 13,708 and 4,216 mtDNA mutations. These and/or other mtDNA mutations may be contributing to the biochemical phenotype. However the main problem with this study is that MRC function in the 11,778 cells prior to fusion was not documented for comparison. There is insufficient evidence from this study alone to conclude that the nuclear environment in 11,778 LHON does not contribute to the biochemical expression of this mutation.
7.6.4 The nature of the nuclear gene(s)

The data presented here suggests that the nuclear background in 3,460 LHON has an influence on the biochemical expression of this mtDNA mutation \textit{in vitro}. The next question to address regards the nature of the nuclear gene(s) that are exerting this influence.

Firstly the host cells used for these mitochondrial transfer studies are transformed cells. Might the lack of expression of a complex I defect in 3,460 LHON merely be a feature of transformed cells? The observation of a clear complex I defect in transformed lymphocytes (lymphoblasts) from 3,460 LHON patients argues against this (Majander \textit{et al.} 1991), although induction of both nuclear and mitochondrial oxidative phosphorylation genes in transformed cells has been observed (Torroni \textit{et al.} 1990).

The second general consideration in this respect is that the biochemical expression of the 3,460 LHON mutation might be a tissue specific phenomenon. The host cell (A549$\rho^0$) is derived from a lung carcinoma cell line and lung tissue may be one in which the 3,460 complex I defect is not expressed even in affected subjects. Presumably nuclear genes would determine such tissue-specificity. LHON is predominantly a tissue-specific disease in terms of clinical symptoms, and this is one of the features of LHON that supports a role of nuclear genes in disease pathogenesis. Undoubtedly tissue specific expression of genes involved in mitochondrial respiratory function does occur. Most available data in this field concerns complex IV subunits where heart and liver specific isoforms of the nuclear encoded subunits VI and VII are well recognized (Lomax and Grossman, 1989; Fabrizi \textit{et al.} 1995), but there is also preliminary evidence for differential expression of complex I subunits between tissues (Clay and Ragan, 1988). It has also been observed that there are tissue specific differences in mitochondrial RNA processing which might account for variation in disease expression (Bindoff \textit{et al.} 1994). Thus it is possible that the restoration of normal complex I activity in cybrids harbouring the 3,460 LHON mutation reflects the presence of a new
"tissue type" environment, and not the lack of a "LHON" nucleus per se. However a complex I defect in 3,460 LHON has now been demonstrated in a variety of clinically unaffected tissues, including lymphocytes (Majander et al. 1991), platelets (Smith et al. 1994), and in this work fibroblasts. In this context a tissue specific effect seems less likely as the sole explanation for differences in biochemical expression between fibroblasts and 3,460-A549p^O cybrids.

The final possibility, and the one that would support many clinical features of LHON is that the LHON subjects carry nuclear gene(s) whose presence is necessary for the complex I defect to be expressed in the presence of the 3,460bp mutation. The observation that complex I activity is normal in cells containing a LHON nucleus but no 3,460 LHON mtDNA demonstrates that both nuclear and mitochondrial abnormalities are required for expression of the complex I defect. MRC function has now been reported on a total of 14 subjects with the 3,460bp mutation (Majander et al. 1991; Howell et al. 1991a; Smith et al. 1994 and this study), all of whom have expressed the complex I defect. This would imply that the nuclear susceptibility gene is not infrequently found, and may even be a common polymorphism. In order to account for the high penetrance it would also need to be dominantly expressed, or X-linked recessive but expressed in affected women through X-inactivation. Candidate genes would obviously include genes encoding complex I subunits, and it is of note that it has been proposed in rodents that some of these reside on the X-chromosome (Day and Scheffler, 1982). As has been previously discussed a "two-hit" model involving an X-linked susceptibility gene and a mitochondrial mutation would best explain the penetrance of LHON (Bu and Rotter, 1991; Harding et al. 1995).

7.6.5 Linking biochemical findings to clinical findings in 3,460 LHON

Both the proband IV_1, and his mother III_2 expressed a biochemical defect in fibroblasts that was complemented in the new nuclear environment. Thus according to the above model both must possess and express the
proposed nuclear genes and mtDNA mutation together. Despite this only IV\textsubscript{1} is to date clinically affected. If this remains the case then these findings infer that possession of both the 3,460 mtDNA mutation and the nuclear factor necessary for its biochemical expression, are still not sufficient to result in clinical disease. The mutant load in the fibroblasts from the proband is marginally higher than that of his unaffected mother, but affected cases with lower mutant loads have been described (Harding \textit{et al.} 1995) making this an improbable explanation. It is also feasible that III\textsubscript{2} will go on to develop symptomatic disease, as she is not yet at the age at which 100\% penetrance is seen. However other studies have also reported subjects homoplasmic for the 3,460 mutation who express the complex I defect, and therefore by implication possess both the nuclear and the mitochondrial mutation, yet who remain clinically asymptomatic (Majander \textit{et al.} 1991; Howell \textit{et al.} 1991a; Smith \textit{et al.} 1994). None have reported a subject with the 3,460bp mutation in whom the complex I defect is not expressed, although there is only data on a total of 14 cases, an insufficient number of subjects from which to make firm conclusions. Documentation of even a single patient with the 3,460 mutation in whom a complex I defect was not expressed would be very informative.

However, given that expression of the complex I defect does not necessarily result in clinical disease, despite the demonstration that the nuclear environment can influence the biochemical expression of the 3,460bp mutation \textit{in vitro}, and the results of pedigree analysis supporting a role for X-linked susceptibility genes, additional mechanisms still must be involved in disease pathogenesis. This will be discussed further in Chapter 8.
Chapter 8. General discussion: mitochondrial abnormalities in the pathogenesis of LHON

Introduction.

The results presented in this thesis have extended information regarding the functional consequences of mtDNA mutations associated with LHON, in particular regarding the 3,460 LHON mutation. Normal electron transfer through complex I has been demonstrated in lymphoblasts with the 11,778bp LHON mutation, and for the first time in fibroblasts bearing the 14,484bp LHON mutation. A polarographic defect in intact mitochondria, as seen in previous studies of 11,778 LHON (Majander et al. 1991), has not been excluded for either mutation. A severe complex I defect has been confirmed in fibroblasts from 3,460 LHON subjects, extending the range of tissues in which the biochemical defect associated with this mutation has been demonstrated as discussed in Chapter 5. However despite this electron transfer defect, fibroblasts bearing the 3,460 LHON mutation are capable of normal ATP production with complex I linked substrates (Chapter 6). The functional inconsistencies between the different LHON genotypes, and that the biochemical defects identified in this and other studies are expressed in both clinically affected and unaffected subjects, must raise doubts about how complex I dysfunction is involved in the pathogenesis of LHON. The possibility that a specific defect in respiratory chain function is not of prime importance, and that other mechanisms might be involved, should therefore be considered.

Preliminary evidence has also been provided (Chapter 7) that the nuclear environment can influence the expression of the biochemical defect in 3,460 LHON. The restoration of complex I activity to control levels in cybrids containing 3,460 mitochondria, but a non-LHON nuclear environment, supports the supposition that nuclear and mitochondrial genes contribute to the biochemical phenotype of a cell, and might be considered in keeping with a "two-hit" model for disease penetrance as suggested by pedigree analysis. However yet again, these results cannot be correlated directly to the clinical
situation, as both affected and unaffected subjects expressed the biochemical defect prior to nuclear complementation, and therefore must have possessed the nuclear factor(s) required for expression of this biochemical phenotype. As previously stated no 3,460 subject, clinically affected or otherwise, has been reported in whom the complex I defect is not expressed (Majander et al. 1991; Howell et al. 1991a; Smith et al. 1994) implying that they too have the necessary nuclear gene(s) - yet 6 of the 14 subjects on whom biochemical data has been documented in the above studies and this thesis, remain clinically unaffected at ages ranging from 22 to 50 years. Thus even possessing both the 3,460 primary mtDNA mutation, and the putative nuclear gene(s) required for expression of the biochemical defect, appears insufficient to result in the clinical disease. As has been observed with other LHON mutations the presence or absence of functional defects, at least in clinically unaffected tissues, does not correlate with the clinical expression of the disease, and their role in disease pathogenesis must be re-addressed, a subject I shall return to.

It is unfortunate in the case of LHON that the tissue primarily affected is not one easy to study. Accessing optic nerve tissue from LHON subjects for studies is ethically and practically prohibitive, and an animal model for LHON does not exist. All of the functional work to date concentrates on clinically unaffected tissues. Although the effects of mtDNA mutations on MRC function in fibroblasts, blood cells or muscle are of interest, it can be argued that conclusions drawn from such studies have no direct bearing on what might be happening in the optic pathways in LHON. In vivo studies on CNS metabolism using magnetic resonance spectroscopy in LHON subjects perhaps represents the approach most likely to be of direct relevance, and have been performed on 11,778 families (Barbiroli et al. 1995). Abnormalities in energy metabolism were demonstrated when compared to controls (discussed in Chapter 5.5.1), but again failed to distinguish between clinically unaffected and affected subjects, and hence have not helped to unravel the questions surrounding mitochondrial dysfunction in the pathogenesis of LHON.
Two broad options exist. Firstly that mitochondrial dysfunction is important, with a tissue specific functional defect confined to the visual pathways in symptomatic individuals, over and above that demonstrated in clinically unaffected tissues. Secondly that mitochondrial dysfunction per se may not be directly related to disease pathogenesis. These options will be considered separately.

8.1 Mitochondrial dysfunction as a pathogenetic factor in LHON.

Before addressing how mitochondrial dysfunction, widely expressed in all tissues studied, might result in a tissue specific clinical condition, one must first consider which particular cell types are primarily affected in LHON. Most of the limited histopathological reports available have supported a primary neuronal degeneration of the retinal ganglion cells and their projections, the optic nerve, with secondary degenerative changes in other parts of the visual system (Kwittken and Barest, 1958; Wilson, 1963; Hume Adams et al. 1966). However all reports are from cases with established optic atrophy due to LHON. Secondary degenerative changes may have taken place, so any conclusions regarding which cells are primarily affected are by necessity speculative. Demyelination confined to the optic nerve, with secondary neuronal loss and gliosis, or a primary vascular or inflammatory process cannot be excluded from these studies. Abnormalities on fundoscopy or in visual evoked potentials in asymptomatic/pre-symptomatic family members (Nikoskelainen et al. 1977; 1982) have been demonstrated, but are non-specific. Pathological data from the visual pathways shortly preceding the onset of significant symptoms, or during the acute phase would be more informative in this respect.

The most recent post mortem study (Kerrison et al. 1995) reported swollen mitochondria with disrupted cristae in the ganglion cells of a woman from the Queensland family with the 14,484 and 4,160bp mutations with isolated blindness. Similar changes are often found in the muscle of patients with mitochondrial myopathies (Fukuhara et al. 1980), in whom there is less
doubt regarding the significance of MRC dysfunction in disease pathogenesis. These findings might represent non-specific post mortem changes, as the authors conceded considerable autolysis had taken place. However calcium containing double-membrane bound inclusions, with rudimentary cristae were also documented in these LHON ganglion cells. These were not found in control tissues, and were thought to represent calcified mitochondrial inclusions resulting from mitochondrial dysfunction. Intracellular calcium accumulation is believed to be a crucial step in cell death induced by excitatory amino acids such as glutamate, and neuronal damage induced by cellular energy depletion may also involve activation of excitatory glutamate receptors (reviewed by Ludolph et al. 1993). Kerrison et al. (1995) suggest that such mechanisms may also be contributing to cell death in LHON, although clearly this is speculative at present.

Assuming the optic nerves are the cells of primary importance in LHON, if MRC dysfunction is of pathogenetic significance in their demise, the question of how some form of complex I dysfunction, expressed in all tissues, might result in the selective death of the optic nerve cells? Either optic nerve cells are particularly susceptible to some dysfunction of complex I that is of less significance in other tissues, or an additional biochemical defect is expressed as a tissue specific phenomenon in these cells.

In considering the first of these possibilities, as the primary LHON mutations identified at positions 11,778, 14,484 and 3,460 are all in genes encoding complex I subunits, it is probable that complex I is in some way involved in disease pathogenesis. Defects in complex I function as demonstrated in 3,460 LHON, the more subtle abnormalities proposed in 11,778 LHON, and potentially present in 14,484 LHON in whom only limited studies have been reported, might differ in detail but all share a secondary common final pathway which results in optic nerve cell death. Discussion on the nature of such secondary mechanisms is purely speculative, but might for instance involve local oxidative stress and/or excitotoxic damage as implicated
in other cell specific neurodegenerations such as Parkinson's disease (Schapira, 1994b), Alzheimer's disease (Mecocci et al. 1994) and Huntington's disease (Beal et al. 1993). Most of the work in this field to date concentrates on Parkinson's disease (PD) in which complex I dysfunction has been implicated (reviewed by Schapira, 1994c). PD is characterized by the selective loss of a very specific cell population in the substantia nigra zona compacta, and although the complex I defect is also most pronounced in this brain region it must also involve the more numerous non-neuronal cells in this region to be readily detected. Controversial evidence has also been presented that this occurs on a background of more widespread MRC dysfunction in PD subjects, as a milder complex I defect has also been identified in platelets (Krige et al. 1992) and in muscle (Cardellach et al. 1993; Blin et al. 1994) from PD patients. Local factors particular to the substantia nigra in PD are believed to contribute to the tissue specificity of this functional defect. These include abnormalities in local oxidative defences with excess free radical production (Jenner et al. 1992; Schapira, 1994b), the local accumulation pro-oxidant substances such as iron and melanin (Good et al. 1992; Mann et al. 1994), and local accumulations by neurotransmitter uptake mechanisms of endogenous or exogenous toxins e.g. catecholamine derivatives (Liptrot et al. 1993) or the opiate derivative MPTP (Adams and Odunze, 1991). Clearly LHON and PD are quite different conditions, and pedigree analyses support a different genetic basis, so whilst their respective aetiological mechanisms are likely to be distinct, one can speculate that a widely expressed functional defect, in the case of LHON consequent upon a mtDNA mutation, might only have pathological consequences in optic nerve cells due to the presence of similar additional local factors. One feature common to both the substantia nigra and the retina is the presence of pigment, implicated in the differential susceptibility of substantia nigra cells in PD by binding metal ions (Hirsch et al. 1988; Good et al. 1992), which in turn contribute to oxidative damage (reviewed by Schapira, 1994b). It is also interesting to note in this context that another extrapyramidal disease
(dystonia) has been associated with LHON in families with a severe complex I defect and a complex I mtDNA mutation at 14,459bp (Shoffner et al. 1995).

The local factor(s) that might render the optic nerve differentially susceptible to minor abnormalities of MRC dysfunction are as yet unidentified. They could be related to a nuclear gene, as pedigree studies would suggest, but environmental agents may equally be involved. A single report of identical twins with the 11,778 mutation, of whom only one is clinically affected and the other remains asymptomatic 6.5 years later, implies that non-genetic factors are involved in the clinical expression of LHON (Johns et al. 1994). The second twin may yet develop visual impairment, but at the very least non-genetic factors such as alcohol intake, smoking and nutrition, must have influence on the age of onset. A mtDNA mutation resulting in tissue specific susceptibility to a normally non-toxic environmental agent has been proposed in Chinese families with aminoglycoside induced deafness. Symptoms in affected individuals occur at low doses of aminoglycosides that would not normally be toxic, associated with a mitochondrial rRNA mutation at position 1555 (Prezant et al. 1993). Prior to the interest surrounding mtDNA mutations and LHON environmental toxins had been proposed as contributory aetiological agents (Hume Adams et al. 1966; Wilson et al. 1971), and perhaps this is an area that should now be re-addressed as studies of MRC function alone have not unravelled the pathogenetic mechanisms in LHON.

A final possibility in this category is that a tissue specific threshold effect exists, such that a reduction of at most 50 - 80% in complex I function in LHON cells is below the threshold at which physiological cell function is impaired in muscle, skin and brain, but in optic nerve cells the threshold is lower and cell death ensues. Differences between tissues in their ability to tolerate specific enzyme defects before oxygen consumption is impaired have been reported, as discussed in chapter 6.5.2.4, although studies in this field are as yet limited in number.
8.2 Pathogenetic factors in LHON unrelated to MRC dysfunction.

A second hypothesis to explain how a tissue specific disease occurs in the context of widespread mitochondrial dysfunction, as in 3,460 LHON, is that MRC dysfunction itself is not involved in the pathogenesis of the disease at all. The mere presence of mtDNA mutations that are confined to LHON families implies that mitochondria do play some role in disease pathogenesis, but it is possible that structural rather than functional differences are important. This hypothesis is supported by the failure to demonstrate any significant reduction in ATP synthesis in 3,460 LHON, and by the normal electron transfer studies observed in 14,484 and 11,778 LHON. Whilst undoubtedly there are abnormalities of mitochondrial function associated with at least two of the three primary LHON mtDNA mutation (3,460 and 11,778), they may be of no clinical significance, as their expression in both clinically unaffected and affected individuals would suggest.

In this context the recent demonstration of circulating auto-antibodies to a human optic nerve protein in LHON patients is particularly relevant (Smith et al. 1995). It is hard to imagine that complex I is not in some way related to disease pathogenesis, given that all the primary mutations so far identified are in genes encoding complex I subunits. Smith et al. (1995) postulated that an auto-immune reaction may be generated through molecular mimicry between abnormal complex I subunit(s) and structural optic nerve protein. The mutant complex I subunit might disrupt the normal assembly of complex I, with mis-targeting of some protein components, leading to their presentation on the cell surface, which would be necessary for such a reaction to occur. Cell surface expression of a mitochondrionally encoded protein has been demonstrated in rodents (Loveland et al. 1990). The microangiopathic appearance of the optic disc in the acute stages of LHON (Nikoskelainen et al. 1983) could indeed represent an inflammatory auto-immune reaction, as was discussed in Chapter 2.8. The occurrence of a multiple sclerosis like illness in some cases (Harding et al. 1992), and the demonstration of positive oligoclonal bands in the CSF of
affected males (Pallini et al. 1988) also support a role for auto-immune mechanisms. The data presented in this thesis sheds no direct light on the possible role of autoimmunity in the pathogenesis of LHON. However that detailed functional studies, including those involving nuclear complementation, have not been able to account for the discrepancies between MRC dysfunction and the clinical expression of the disease does indicate that other avenues such as autoimmunity should be pursued.

8.3 Future work in the study of LHON

8.3.1 Further studies of MRC function in LHON

The role of complex I dysfunction in the pathogenesis of LHON is not yet clear. It may transpire that MRC functional defects are not of primary importance in this disease, as previously discussed, but even if this is the case further studies of complex I function in LHON subjects may enhance our understanding of the normal functioning of this complicated enzyme. The number of subjects studied to date, particularly with the 14,484 mutation is very small. Enzyme analysis, polarography and data on ATP synthesis should be extended to include more LHON subjects with this mutation, both affected and non-affected. Polarographic studies and assays of ATP synthesis on intact mitochondria from cells possessing the 14,484 mutation would be particularly important as no such data has yet been reported, except in a family where the 4,160bp has not been excluded (Oostra et al. 1995).

Following the demonstration in this work of normal ATP synthesis in cells with the 3,460bp mutation, expressing a complex I electron transfer defect, other approaches to the study of intact mitochondrial function in 3,460 LHON subjects should be considered in order to validate the findings presented in Chapter 6. Firstly, although the ATP synthesis was inhibited in parallel to incremental complex I inhibition induced by the inhibitor rotenone in control subjects, study of other subjects with established mitochondrial diseases and MRC dysfunction using the same methodology would help to establish the
sensitivity and specificity or this assay. Polarographic analysis of intact mitochondria in other studies (Larsson et al. 1991; Majander et al. 1991) have consistently indicated that complex I function is reduced in 11,778 LHON subjects studied (total number of subjects = 6), so if reduced ATP synthesis with complex I linked substrates could be demonstrated in these subjects this would add weight to the evidence for significant MRC dysfunction in 11,778 LHON, as well as supporting the sensitivity of the assay method. MR spectroscopic studies of muscle and brain function in 3,460 and 14,484 LHON, such as have been reportedly abnormal in 11,778 LHON subjects (Cortelli et al. 1991), would also be of interest in establishing the presence or absence of significant mitochondrial dysfunction in vivo. Other approaches might also be evaluated such as fluorimetric measurements of mitochondrial membrane potential in cells with LHON mtDNA mutations. This approach has proved possible in living cells by using a J-aggregate-forming dye (JC-1; Smiley et al. 1991), which rapidly aggregates in certain local environments and displays "resonance fluorescence". Local aggregation is proportional to the mitochondrial membrane potential, whose maintenance is in turn dependent oxidative phosphorylation. The discriminatory value of such dyes in the study of mitochondrial diseases has not yet been established.

Finally more detailed studies of complex I function are needed in 3,460 LHON in order to explain how normal ATP synthesis with complex I linked substrates despite a severe electron transfer defect is possible. Kinetic studies with respect to NADH should be pursued in order to establish the $K_m$, although this would require a large mitochondrial yield and/or a more pure mitochondrial preparation in order to obtain replicate values for each data point. The implication from inhibitor studies that there are more than two electron transfer paths through complex I is also of particular interest (Anderson and Trgovcich-Zacok, 1995). Should new complex I inhibitors become available their effects on complex I activity in 3,460 LHON and controls might shed further light on this hypothesis. Electron spin resonance studies can demonstrate the
oxidized/reduced state of the Fe-S clusters in complex I under different conditions (Vinogradov et al. 1995). Comparison of the patterns in 3,460 subjects with those of controls might also provide further clues to the presence or absence of additional electron transfer pathways.

The potential secondary consequences of mitochondrial dysfunction, including a search for increased free radical production and oxidative damage should also be investigated in cells harbouring the LHON mutations.

**8.3.2 Studies of complex I protein composition in LHON**

Although the data presented in this thesis (Chapter 6.5.1-2) did not detect any abnormalities in the subunit composition of complex I in 3,460 LHON fibroblasts, this is only preliminary: generally weak signals were obtained due to the use of only partially enriched mitochondrial fractions, and information regarding the mitochondrially encoded subunits was limited, although mitochondrial translation studies were normal. More information might be provided using antibodies generated against human complex I. Bentlage et al. (1995) have generated monoclonal antibodies against synthesized peptides whose sequence has been based on computer predicted epitopes of mitochondrial subunits. Western blots using such antibodies to the mutant complex I subunits in LHON (ND1, ND4 and ND6), compared to controls, if normal would support the data already presented.

Applying the same principles mutant complex I peptides generated from computer predicted sequences corresponding to the LHON mtDNA mutations could be used to screen serum from LHON subjects for crossreacting antibodies. Identification of such antibodies would provide support for a pathogenetic model in which autoantibodies cross react with abnormally expressed mitochondrial proteins in LHON (Smith et al. 1995). Such a model might also be supported by demonstrating an acute inflammatory response as part of the early disease process for instance by measuring acute phase proteins in the serum, or more probably in the CSF, of newly presenting LHON
subjects, although in practical terms this would be difficult to pursue, certainly in terms of the CSF. Finally quantification of oxidative phosphorylation enzyme proteins using blue native electrophoresis and two-dimensional resolution as described by Schägger (1995) would allow a comparison of complex I protein levels relative to other MRC enzymes to be made in tissues/cells from LHON subjects and controls, and consideration of these results in relation to complex I activities.

8.3.3 Nuclear complementation studies in LHON

The number of reported LHON pedigrees worldwide must now be of sufficient size to consider twin studies in affected families. To date there has been only a single twin case report, with discordance for symptomatic disease in identical twins (Johns et al. 1994). Review of all the reported pedigrees and comparison of symptomatic concordance and discordance amongst siblings, non-identical and identical twins would shed light on the relative contribution of nuclear genetic factors to the pathogenesis of LHON, if there are sufficient study numbers available.

Nonetheless some contribution from nuclear genes is well supported by the pedigree analyses of (Bu and Rotter, 1991; Harding et al. 1995). The data showing nuclear complementation of complex I dysfunction in 3,460 LHON presented in this thesis (Chapter 7) must be extended in order to draw firm conclusions. In all subsequent mitochondrial transfer experiments proposed, quantification of absolute mtDNA levels in $p^0$ derived cybrids should be performed to exclude any influence this may have on the interpretation of results. A positive control experiment should be undertaken in order to establish that a complex I defect can be expressed in 3,460-$p^0$ cells in the presence of the required nuclear gene(s). This would involve transforming 3,460 LHON fibroblasts, rendering them $p^0$, and re-introducing 3,460 LHON mtDNA. The complex I defect would be expected to persist in such a system assuming that the proposed LHON nuclear factor was not affected by the process of mtDNA
depletion with ethidium bromide. Secondly 3,460 mitochondrial transfer studies should also be undertaken using transformed control fibroblasts that have been rendered \(\rho^0\), in order to exclude the possibility that complex I function has been restored to 3460-A549\(\rho^0\) cells purely as a result of a tissue-type change. Finally transfer of 3,460 mitochondria into transformed LHON cells with either the 11,778bp mutation, or the 14,484bp mutation that have been depleted of their own mtDNA would establish whether the nuclear factor present in 3,460 LHON necessary for the complex I defect to be expressed is also present in LHON subjects with other mutations.

If the above studies supported a role for nuclear genes in the expression of biochemical dysfunction associated with LHON it would be important to work towards identifying the nuclear gene(s) responsible. Linkage analysis in LHON has so far not been rewarding, though it has so far been confined to the X-chromosome as penetrance patterns have indicated. However it is possible that genes on other chromosomes are important in the expression of LHON, although their expression might in turn be related to X-linked elements. If the nuclear gene in 3,460 LHON subjects necessary for reduced complex I activity to be detected is recessive (in which case it is probably on the X-chromosome), it might be possible to confirm which chromosome is responsible for nuclear complementation by micro-cell fusion studies (Lugo et al. 1987). This allows specific chromosomes, individually or in small groups, to be introduced into primary 3,460 cells, yielding hybrids which will contain non-LHON chromosome(s), in addition to the complete nucleus of a LHON subject, and mitochondria with the 3,460bp mutation. Subsequent MRC function analysis would aim to identify those cybrids in which nuclear complementation of complex I dysfunction had occurred, followed by cytogenetic analysis to establish which additional chromosome(s) has been inserted into the hybrids. From a number of such experiments it would be possible to determine which non-LHON chromosome was necessary for complementation to occur.
8.3.4 Final comments

Many questions regarding the role of mitochondrial mutations in the pathogenesis of LHON remain unanswered, and although preliminary evidence supporting a role for nuclear factors in the biochemical expression of one primary LHON mutation has been presented, further studies are required to confirm this data and interpret it reliably. Studies directed at further characterization of mitochondrial function in LHON subjects must continue, but other avenues should also be addressed. Of particular importance to our understanding will be the identification of molecular or biochemical features able to distinguish between clinically affected from unaffected individuals.
Chapter 9. Mitochondrial changes in fibroblasts from ageing donors

Introduction:

One theory of ageing proposes a key role for mitochondria in the ageing process (Fleming et al. 1982). The data in support of this has already been reviewed in Chapter 3.4. Briefly, Fleming et al. (1982) proposed that the occurrence of mtDNA mutations increased with age, accompanied by a decline in MRC enzyme function, and that the consequent fall in cellular energy production would ultimately result in cell death. Such a process occurring gradually through life might account for many of the changes accepted as part of the normal ageing process (Chapter 3.1), and contribute to age-related diseases. Observations in support of this hypothesis include the accumulation of deleted mtDNA in several tissues (Chapter 3.4.1), and a fall in MRC activities (Chapter 3.4.2) with increasing age. However with the exception of one study (Hayashi et al. 1994a), most work has been performed on tissue specimens, such as muscle biopsies or post-mortem muscle/brain samples, and not in a tissue culture model. This Chapter reports MRC function and mtDNA analysis in cultured fibroblasts from a wide age-range of healthy subjects. MtDNA studies were confined to identification of the mtDNA mutation that has been most frequently observed in ageing subjects, the "common" (4,997bp) deletion.

9.1 MtDNA in cultured fibroblasts from ageing donors

DNA was extracted from cultured fibroblasts (passage number 10) from 13 healthy donors aged 9 - 77 years. PCR as described in Chapter 4.3.3 was used to amplify a 351bp fragment from mtDNA bearing the 4,997 common deletion (Figure 34A). PCR was also carried out in parallel on a set of serially diluted DNA samples with a known percentage of mtDNA with the common
deletion (Figure 34B). Using this as a visual reference the 13 healthy donors were estimated to have between 0.001% and 0.01% mtDNA with the common deletion. There were no apparent major differences in the levels of deleted molecules in cultured fibroblasts from young (9 years) or old (77 years) donors. PCR analysis of DNA from an HGPS subject (DP) was conducted in parallel to the controls, but this data will be represented and discussed in chapter 10.

That the 351bp product was amplified from mtDNA possessing the common deletion was confirmed by the presence of an *Xba* 1 restriction site in the PCR product, which was digested into 251 and 100bp fragments as shown in Figure 35.

### 9.2 MRC function in cultured fibroblasts from ageing donors

Spectrophotometric analysis of MRC enzymes was carried out on MEFs prepared from cultured fibroblasts (at p = 7 - 13) from 13 healthy donors aged 9 - 77 years. Linear regression analysis was performed and two-tailed p values calculated using the Spearman rank correlation coefficient. The results are shown in Table 16 and Figure 36. A significant negative correlation between COX specific activity and increasing age was observed. When expressed as a CS ratio an age related decline was observed for both complex I and COX activities, both of which gave significant correlation coefficients. The complex I:CS ratio in a 77 year old (0.1341) subject was 68% that of subjects under 25 years (complex I:CS ratio 0.1966; n = 5, mean age = 17.2 years). Complex IV:CS at 77 years (0.0159) was only 61% that of the younger subjects (0.0257). There were insufficient numbers to perform statistical comparisons between age groups. An age-related fall in complex II/III function was also seen, but this change was not significant. Citrate synthase activity did not change significantly with age.
Figure 34. PCR amplification products from mtDNA possessing the common deletion, separated on a 2% agarose gel. A: PCR products from control fibroblasts labelled according to donor age in years. DP = HGPS patient, age 20 years, and Δ = positive control calculated to have 80% deletion on southern blotting. M = θ 174 Hinf I marker. B: Serially diluted DNA containing known percentages of mt DNA with the common deletion. The percentage of deleted molecules is indicated beneath each band. H = water blank.
Figure 35. Xba I restricted (R), and unrestricted (U) PCR products from a patient known to possess mtDNA with the common deletion (Δ), confirming the presence of an restriction site.
Table 16. Correlation of MRC enzyme activities with age in fibroblast MEFs from controls aged 9 - 77 years.

A: Specific activity

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Spearman correlation coefficient</th>
<th>95% confidence limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS</td>
<td>-0.06</td>
<td>-5.68 to 5.60</td>
</tr>
<tr>
<td>NADH CoQ₁ R</td>
<td>-1.98</td>
<td>-6.85 to 4.12</td>
</tr>
<tr>
<td>SCcR</td>
<td>-1.01</td>
<td>-5.71 to 5.56</td>
</tr>
<tr>
<td>COX</td>
<td>-5.72*</td>
<td>-8.59 to -0.13</td>
</tr>
</tbody>
</table>

B: CS Ratios

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Spearman correlation coefficient</th>
<th>95% confidence limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH CoQ₁ R</td>
<td>-5.82*</td>
<td>-8.63 to 0.278</td>
</tr>
<tr>
<td>SCcR</td>
<td>-2.80</td>
<td>-7.51 to 2.93</td>
</tr>
<tr>
<td>COX</td>
<td>-8.02**</td>
<td>-9.41 to -4.35</td>
</tr>
</tbody>
</table>

A: specific activities are expressed as nmols/min/mg protein except for COX where the value is k/min/mg protein. B: data expressed as a ratio to citrate synthase activity. * p <0.05; ** p = 0.001
Figure 36. The effect of donor age on MRC enzyme activities in MEFs from cultured fibroblasts of healthy donors. A-CS specific activity; B-complex I:CS ratio; C-complex II/III:CS ratio; D-complex IV:CS ratio. Each point represents the mean from triplicate preparations from a healthy control subject. Two-tailed p values have been calculated using the Spearman Rank correlation coefficient.

9.3 Discussion: Mitochondrial changes in cultured fibroblasts from ageing donors

9.3.1 Selecting subjects to study biological ageing

A major difficulty in selecting subjects in whom to study the effects of ageing is defining which, if any, disease states are acceptable as part of the ageing process. It is generally believed that many conditions such as atherosclerotic vascular disease, neurodegenerative conditions and diabetes, have an increasing prevalence with age as discussed in Chapter 3.1. Some studies on ageing have used the existence of any disease state as an exclusion criteria (Skelton et al. 1994), whilst others go further and have carefully matched physical activity levels in order to rule out potential confounding factors (Brierly et al. 1995). The potential effects of exercise levels on MRC function is an appropriate consideration when muscle is the tissue of study, but
less likely to have a significant effect on MRC function in fibroblasts. In addition one potential problem with such careful selection strategies may be that in some cases elderly individuals with activity levels similar to that of younger controls may be considered "super-fit", and not representative of "normal" ageing, in that most people become less active, have minor cognitive decline, and evidence of changes such as atherosclerosis as they reach old age.

The study presented in this thesis did not impose such rigorous selection criteria. None of the subjects were smokers or taking any medication that might affect mitochondrial function, and those up to and including the age of 50 years had no significant medical history. The two older subjects had conditions common in old age (mild ischaemic heart disease in the 67 year old subject, and diabetes with a peripheral neuropathy in the 77 year old subject). This spectrum of subjects was considered representative of a normal ageing population, though larger numbers would have been preferable.

9.3.2 MtDNA in fibroblasts from ageing donors

Previous studies have documented increased prevalence, and an increase in detectable levels of the common deletion in tissues from ageing subjects (Cortopassi and Arnheim, 1990; Linnane et al. 1990). Most groups have used post mortem muscle or brain samples, on the assumption that these tissues are more susceptible to oxidative damage by virtue of their high metabolic demands. If mtDNA damage is related to oxidative stresses mutations might be more readily observed in these tissues than others (Ames, 1989). An increased prevalence and quantity of mtDNA with the common deletion with age has also been documented in skin biopsies from healthy living donors (Yang et al. 1994), though more so in sun-exposed skin than that shielded from potentially harmful ultra-violet rays (Yang et al. 1995) implying environmental factors may also play a role in the pathogenesis of mtDNA mutations. Deleted mtDNA has not previously been demonstrated in cultured cells from healthy ageing donors, though only a single study has previously
addressed this: Hayashi et al. (1994a) were unable to detect the common deletion in cultured fibroblasts from ageing donors by PCR with a sensitivity of 0.02% (1 in 5,000 mtDNA molecules), or to detect other large-scale mtDNA rearrangements on southern blots. The sensitivity of PCR in this study was greater (0.001% - 1 in 100,000 molecules) which may explain why low levels of deleted mtDNA were demonstrated even in the youngest subjects aged 9 and 11 years. Deleted mtDNA is not always maintained in rapidly dividing cells in vivo (Bernes et al. 1993), or in tissue culture even when the level in non-dividing cells is very high (Holt et al. 1988). In clonal myoblast cultures from patients with high proportions of deleted mtDNA, cells heteroplasmic for the deletion grow slower than those with wild-type mtDNA (Moraes et al. 1989b), and it has been proposed that any replicative advantage that the smaller deleted mtDNA molecule might have over wild-type mtDNA (Larsson et al. 1990) is outweighed by the functional disadvantage conferred on the dividing cell. That at low levels (estimated < 1 in 1,000 mtDNA molecules) the deletion is persisting in culture in this study perhaps suggests that in itself it does not have significant disadvantageous functional consequences.

The methodology used in this study at best only allows limited quantitative estimations. Thus other than confirming the presence of low levels of deletion in all subjects, firm conclusions regarding absolute levels or potential changes with ageing cannot be made. However the results are in keeping with previous ageing studies which suggest that at most deleted mtDNA represents < 1 in 1,000 molecules (Yen et al. 1991; Cortopassi et al. 1992; Baumer et al. 1994). Therefore any functional changes subsequently demonstrated may not be considered consequent solely upon the presence of accumulated mtDNA bearing the common deletion as has previously been proposed (Hattori et al. 1991; Linnane et al. 1992). In specific mitochondrial disease states associated with the common deletion, such as CPEO, mtDNA bearing the common deletion must represent as much as 60% of total mtDNA before mitochondrial function is significantly compromised (Hayashi et al. 1991; Fassati et al. 1994).
The presence of other mtDNA mutations that together with the common deletion might constitute a significant mutational load cannot be excluded. Other than the primary LHON mutations (11,778bp, 14,484bp, 3,460bp; data not shown) further mtDNA analysis was not performed on these controls. Several studies have found low levels of different mutations accumulating with age including other deletions (Hsieh et al. 1994) and D-loop duplications (Lee et al. 1994). Others have documented the occurrence of a point mutation as an age associated event (Münscher et al. 1993; Zhang et al. 1993). Hence it is possible that the common deletion in ageing tissues represents only a small fraction of the total mtDNA mutations. The contribution of such mutations to functional changes remains unclear.

9.3.3 MRC function and ageing

The data from this study is broadly in agreement with the existing literature, demonstrating a marked decline in COX activity with age in a variety of tissues, and a smaller, though still significant, fall in complex I activity. The many existing studies addressing functional MRC changes with age are summarized in Table 17.

The majority of work to date has been carried out on skeletal muscle samples. Skeletal muscle performance in terms of speed and strength is known to decline with age (Larsson et al. 1979), and it is also a tissue highly dependant on oxidative metabolism so might be expected to exemplify any age related changes in MRC function. However because of the relative invasiveness of many biopsy procedures, samples are most commonly obtained from individuals undergoing orthopaedic surgery. This causes a potential bias in patient selection. Children are likely to come to surgery as a result of congenital deformities or accidents (Lefai et al. 1995), young adults most commonly the result of trauma, whilst in the elderly the commonest subjects are those with a fractured femur or requiring hip replacement (Cooper et al. 1992b), often co-existent with general frailty and inactivity. Controlling adequately for variables
### Table 17. Reports of MRC function in ageing

<table>
<thead>
<tr>
<th>First author</th>
<th>Year</th>
<th>Tissue</th>
<th>n (age range)</th>
<th>Methodology*</th>
<th>MRC defect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lefai</td>
<td>1995</td>
<td>skeletal muscle</td>
<td>43 (4 - 19 years)</td>
<td>spec. assays</td>
<td>complex IV</td>
</tr>
<tr>
<td>Rifai</td>
<td>1995</td>
<td>skeletal muscle</td>
<td>28 (22 - 77 years)</td>
<td>histochemistry (complex IV only)</td>
<td>complex IV</td>
</tr>
<tr>
<td>Blin</td>
<td>1994</td>
<td>skeletal muscle</td>
<td>43 (40 - 87 years)</td>
<td>spec. assays</td>
<td>complex I</td>
</tr>
<tr>
<td>Hsieh</td>
<td>1994</td>
<td>skeletal muscle</td>
<td>45 (15 - 80 years)</td>
<td>spec. assays</td>
<td>complexes I &amp; IV</td>
</tr>
<tr>
<td>Hayashi</td>
<td>1994a</td>
<td>cultured fibroblasts</td>
<td>16 (0 - 97 years)</td>
<td>spec. assays (complex IV only)</td>
<td>complex IV</td>
</tr>
<tr>
<td>Bowling</td>
<td>1993</td>
<td>monkey brain</td>
<td>20 (5 - 34 years)</td>
<td>spec. assays</td>
<td>complexes I &amp; IV</td>
</tr>
<tr>
<td>Di Monte</td>
<td>1993</td>
<td>monkey brain</td>
<td>18 (3 - 16 years)</td>
<td>ATP synthesis</td>
<td>complexes I - V</td>
</tr>
<tr>
<td>Cardellach</td>
<td>1993</td>
<td>skeletal muscle</td>
<td>10 (18 - 69 years)</td>
<td>spec. assays and polarography</td>
<td>none</td>
</tr>
<tr>
<td>Cooper</td>
<td>1992b</td>
<td>skeletal muscle</td>
<td>14 (21 - 78 years)</td>
<td>spec. assays and polarography</td>
<td>complexes I &amp; IV</td>
</tr>
<tr>
<td>Kish</td>
<td>1992</td>
<td>brain</td>
<td>30 (69 +/- 2 years)**</td>
<td>spec. assays (complex IV only)</td>
<td>none</td>
</tr>
<tr>
<td>Van Zuylen</td>
<td>1992</td>
<td>platelets</td>
<td>13 (26 - 87 years)</td>
<td>spec. assays (complex IV only)</td>
<td>complex IV</td>
</tr>
<tr>
<td>Muller-Höcker</td>
<td>1990</td>
<td>skeletal muscle</td>
<td>115 (0 - 97 years)</td>
<td>histochemistry (complex IV only)</td>
<td>complex IV</td>
</tr>
<tr>
<td>Müller-Höcker</td>
<td>1989</td>
<td>cardiac muscle</td>
<td>149 (0 -97 years)</td>
<td>histochemistry (complex IV only)</td>
<td>complex IV</td>
</tr>
<tr>
<td>Yen</td>
<td>1989</td>
<td>liver</td>
<td>35 (31 - 76 years)</td>
<td>polarography</td>
<td>complexes I to IV</td>
</tr>
<tr>
<td>Trounce</td>
<td>1989</td>
<td>skeletal muscle</td>
<td>29 (16 - 92 years)</td>
<td>spec. assays and polarography</td>
<td>complex IV</td>
</tr>
</tbody>
</table>

* spec. assays = spectrophotometric assays of citrate synthase and complexes I - IV unless otherwise stated; **Mean age +/- SD. Range not given.
which could conceivably affect MRC function, such as general debility, level of activity and nutritional status, is difficult. Interpretation of studies on MRC function in muscle must therefore be guarded. As a group the most consistent finding is one of a decline in COX with age, which has been shown using both polarographic and spectrophotometric techniques in orthopaedic muscle biopsies (Trounce et al. 1989; Cooper et al. 1992b; Hsieh et al. 1994) as well as in needle biopsy specimens from healthy donors (Rifai et al. 1995). In keeping with this histochemical staining of post-mortem samples from skeletal (Muller-Höcker, 1990) and cardiac (Müller-Höcker, 1989) muscle reveals an increasing prevalence and proportion of COX negative fibres with age, reaching a maximum of 0.47% fibres affected in diaphragmatic muscle. It can be seen from Table 17 that those studies that have not observed this relationship between COX and age have by enlarge used small control ranges (Kish et al. 1992), or small numbers of subjects (Cardellach et al. 1993). However it should also be stated that these particular studies were primarily designed to address MRC function in disease states, namely Alzheimer's and Parkinson's disease respectively, rather than to address the relationship between age and MRC function. None the less the authors did comment on their data with regard to the relationship between ageing and MRC function and have thus been included in this summary.

A decline in COX activity with normal ageing is further supported by studies on other tissues such as liver (Yen et al. 1989), platelets (Van Zuylen et al. 1992) and cultured fibroblasts (Hayashi et al. 1994a).

The majority of studies in which complex I activity has been examined have also observed an age related decline in activity, both by polarographic assessment (Cooper et al. 1992b; Hsieh et al. 1994) or enzymatic analysis (Blin et al. 1994). Hsieh et al. (1994) did see a fall with age in complex I electron transport activity in muscle mitochondria, but it was mild in comparison to that seen in COX. Formal regression analysis was not documented. In many of the remaining ageing studies listed in Table 17, complex I function was not
specifically assessed. Whilst histochemical analysis of muscle is commonly confined to COX and succinate dehydrogenase staining, and thus does not assess complex I function as in the studies by Müller-Hocker (1989,1990) and Rifai et al. (1995), in other studies spectrophotometric assays were only performed for citrate synthase and COX activities for reasons that are not clear. Of those in which complex I activity was measured, only two studies did not observe a significant change with age, though a fall in COX activity was clearly demonstrable in their study populations (Trounce et al. 1989; Lefai et al. 1995). As discussed in 9.4.1 any work of this kind has potential limitations. However the subjects studied in this thesis are considered representative of the "normal" ageing process as far as is possible with such small numbers. Furthermore, in possible contrast to muscle, one may not expect MRC function in cultured fibroblasts to be influenced by activity levels of the subject. The majority of the studies on ageing have studied muscle or brain, but if MRC dysfunction is a key feature of the ageing process, a process which also clinically affects the skin and connective tissues, defects should be detectable in other tissues, including fibroblasts. In addition one advantage of using cultured cells is that culture conditions should be identical for all samples. Thus nutritional and neuroendocrine variables within the donor subjects should be largely excluded, though factors that might pre-condition cells and have an irreversible effect, may still have an influence. This work therefore provides further evidence that a decline in MRC function occurs with age, and that this particularly affects COX and complex I activities.

9.3.4 The contribution of nuclear and mitochondrial genomes to age related MRC dysfunction

The previous section has demonstrated that much evidence exists for an accumulation of mtDNA mutations (chapter 9.3.2), and a decline in MRC function (chapter 9.3.3) with age. The mitochondrial theory of ageing was proposed before much of the supportive data was available (Fleming et al.
1982), and linked these two groups of observations. Fleming et al. (1982) proposed that damaged mtDNA increased with time, through a combination of oxidative damage, spontaneous mutation and poor repair capacity. This ultimately would give rise to impaired MRC function. Dysfunctional electron transfer itself could then further contribute to local damage by way of free radical production. The end result would be a reduced energy capacity of the cell, defective physiological functioning and ultimately cell death (Linnane et al. 1989; Wallace, 1995).

However in a study by Hayashi et al. (1994), mitochondria (containing mtDNA) from ageing donors, expressing a defect in complex IV activity, were placed in a new "young" nuclear environment (transformed \( \rho^0 \) HeLa cells). The resultant fusion cybrids had COX activities comparable to those of young donors, intimating that the "old" mitochondria were functionally intact, and therefore that nuclear factors must be responsible for the functional decline seen in fibroblasts from aged donors. In addition placing the "young" \( \rho^0 \) HeLa nucleus into intact fibroblasts from ageing donors restored COX activities to "young" levels. These hybrid cells contained nuclear genes from both the ageing donors and the \( \rho^0 \) HeLa cells, so that the COX defect was no longer observed implies that the proposed nuclear genes(s) responsible in the ageing fibroblasts was recessive in nature. Although this is only a single study, and a relatively small number of cybrid clones were analysed, the hypothesis that MRC dysfunction associated with ageing might not be dependant on the presence of mutated mtDNA is an interesting one. As was reviewed in Part 1, nuclear genes not only contribute the majority of the respiratory chain protein subunits (Chapter 1.2), but are also involved in the replication, transcription and translation of mtDNA, as well as in a regulatory role (Chapter 1.1). The contributory role of mtDNA in the changes in MRC function seen with ageing is far from proven, and may prove to be a secondary marker of mitochondrial damage rather than a primary pathogenetic event. Furthermore, although a model of ageing in which mitochondrial dysfunction might play a primary
aetiological role, and directly contribute some of the clinical features, is conceivable, mitochondrial dysfunction might itself be a secondary marker of alternative changes. A primary role for mitochondria in the ageing process remains unproven.
Chapter 10. Mitochondrial studies in Hutchinson-Gilford progeria syndrome (HGPS)

Introduction.

This chapter presents data on the role of mitochondrial dysfunction in HGPS. As reviewed in Chapter 3.6 HGPS shares several clinical features with normal ageing. Although it cannot be regarded as simply accelerated ageing, the clinical similarities suggest there may be common pathogenetic mechanisms. The consistent clinical phenotype of HGPS supports a genetic basis for the condition, however the cause of HGPS remains unknown. Despite the increasing interest in the relationship between mitochondrial dysfunction and normal ageing in recent years, detailed mitochondrial analyses in HGPS have not been reported. This chapter will report studies on two patients with HGPS, DP (age 20 years) and HG1 (age 16 years), performed in order to ascertain whether mitochondrial dysfunction and/or mutations of mtDNA might be associated with, or contribute to, the pathogenesis of this condition.

10.1 Growth characteristics of HGPS fibroblasts

Growth curves were not performed on HGPS cells as only limited cellular material was available and reserved for molecular and functional analysis. However fibroblasts derived from patients with HGPS grew slower in culture and showed signs of cellular senescence at earlier passages than controls. This was particularly true in the case of HG1, limiting further study of this subject. It took 44 days for DP fibroblasts (n=1) to approach confluence after 4 passages, but only 18 - 22 days for controls (n = 5). HG1 fibroblasts ceased to divide and started to die off after passage number 8 - 10, and DP fibroblasts after 15 - 20 passages. Control fibroblasts from donors of any age were still healthy up to passage 30.
10.2 MtDNA in HGPS fibroblasts

PCR analysis on DNA extracted from DP and HG1 using primers for the mtDNA "common" deletion amplified a product of comparable intensity to those found in age matched controls on three separate occasions. A sample result is shown in Figure 37 where DP, aged 20, is situated between two age matched controls.

![Figure 37. PCR products from HGPS and control fibroblasts using primers for mtDNA with the common deletion, separated on a 2% agarose gel. The controls are labelled according to their age in years. M = 0174 Hae III marker; DP = HGPS patient, age 20 years, and Δ = positive control calculated to have 80% deletion on southern blotting.](image)

10.3 MRC function in HGPS fibroblasts

Spectrophotometric analysis of MRC enzymes was performed on mitochondrial fractions prepared from cultured fibroblasts, harvested at passage numbers 8 - 12, from 2 HGPS patients (DP age 20; HG1 age 16), and 6 controls (age 21 to 32 years). The results are shown in Table 18 and Figure 38. There was a significant decrease in both COX activity (57% decreased) and complex II/III activity (49% decreased) compared to controls when expressed as a CS ratios. The complex II/III and IV CS ratios in HGPS subjects all fell outside the control range, being lower even than the activities recorded from the oldest control studied in Chapter 10. (Age 77 years; complex II/III:CS ratio
0.3372; complex IV:CS ratio 0.0159). Both defects were also apparent when results were expressed as specific activities. There was no significant difference in CS or in complex I activity in the HGPS patients compared to controls.

Figure 38. MRC enzyme activities in MEFs from control and HGPS fibroblasts. Activities are expressed as CS ratios, and the combined means (+/-SD) from 6 controls (open bars) and 2 HGPS patients (shaded bars) are shown.
<table>
<thead>
<tr>
<th></th>
<th>A: Specific activities</th>
<th>B: Citrate synthase ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CS</td>
<td>Complex I (X10)</td>
</tr>
<tr>
<td>Controls (n = 6)</td>
<td>101.4 +/- 21.4</td>
<td>12.4 +/- 4.2</td>
</tr>
<tr>
<td>HGPS : DP</td>
<td>104.4 +/- 26.7</td>
<td>13.9 +/- 4.0</td>
</tr>
<tr>
<td>HG1</td>
<td>125.7</td>
<td>19.3</td>
</tr>
<tr>
<td>Mean</td>
<td>115.0 +/- 15.1</td>
<td>16.6 +/- 3.8</td>
</tr>
</tbody>
</table>

Mitochondrial enzyme activities in MEFs from age matched controls and two patients with HGPS (DP, HG1). A: specific activities are expressed as nmols/min./mg protein except for complex IV which is expressed as the first order rate constant k/min/mg protein. B: data from A expressed as a ratio to CS activity. Mean results of three separate preparations from each control have been combined prior to analysis, and the values given are means +/- SD. The value for DP is the mean of triplicate preparations. For HG1 only sufficient material for one preparation was generated. * p < 0.05; **p < 0.001, calculated from the combined means of the two HGPS patients compared to those of the 6 controls. Statistical analysis was carried out using a non-parametric t-test modified for small sample numbers (Parker, 1979).
10.4 COX western blots in HGPS

Western blots of protein extracts from DP and 2 age matched controls, using a cocktail of monoclonal antibodies against COX subunits, revealed no apparent differences in the mitochondrial-encoded COX subunits I or II, nor in the nuclear-encoded subunit IV. The signal obtained on a separate occasion using an anti-porin antibody was comparable in all three subjects, indicating no significant difference in mitochondrial protein loading (Figure 39).

Figure 39. Western blots using a cocktail of monoclonal antibodies directed against COX subunits I, II and IV, distinguished on the basis of their relative mobilities (Capaldi et al. 1995). The same blot was probed with a porin antibody (31kDa; Konstantinova et al. 1995) on a separated occasion (top gel). Protein extracts from fibroblasts at passage 12 were separated on 15%-SDS-PAGE containing 6M urea and blotted onto PVDF membrane. Bound antibody has been detected using chemiluminescence. Subjects shown are two age-matched controls (C) and an HGPS patient (DP). M kDa = molecular weight marker positions.
10.5 Mitochondrial translation studies in HGPS

*In vitro* mitochondrial translation studies revealed no qualitative differences in mitochondrially encoded proteins between an HGPS subject (DP) and an age-matched control (Figure 40). Mitochondrial protein synthesis in the HGPS cells generated a similar pattern of labelled products to that observed in the control. Although not all of the known mitochondrial translation products were detected on this autoradiograph, the polypeptide pattern resembles that previously seen in cultured skin fibroblasts (Bodnar *et al.* 1995). Protein synthesis was not observed in the presence of chloramphenicol.

Equivalent radioactive counts from labelled HGPS and control samples were been loaded on the gel shown, in order to correct for any variation in size of the original labelled cell pellet or of MEF enrichment. However equivalent counts were obtained from similar volumes of labelled mitochondria on three separate occasions, indicating there was no significant quantitative difference in mitochondrial protein synthesis between HGPS and controls.

10.6 Nuclear complementation of mitochondrial dysfunction in HGPS

10.6.1 Enucleation conditions and confirmation of successful fusion

MRC activities were not detected in the A549ρ 0 cells (Chapter 7.1), and therefore these cells were a suitable line in which to study the effects of a new nuclear environment on the mitochondrial defects identified in HGPS. Efficient enucleation of fibroblasts (Chapter 4.2.7) from DP was achieved by centrifugation at 25,000g_{max} for 20 minutes at 350°C. A total of 16 DP-A549ρ 0 cybrid colonies were isolated of which 13 survived long enough in culture for mtDNA analysis; 3 colonies were lost to infection prior to this. PCR analysis confirmed the presence of mtDNA in all these cybrid colonies (Figure 41). Control cybrid colonies were obtained from fibroblasts from a 22 year old control as in Chapter 7.
Figure 40. $^{35}$S labelled mitochondrially synthesized proteins from control (C) and HGPS (DP) fibroblasts separated on a 16.5%-SDS-PAGE containing 6M urea. M kDa = molecular weight marker positions. Equal counts were loaded in each lane. Individual subunits have been identified on the basis of their relative mobilities as ND5 (apparent Mf 51kDa), COXI (44kDa), ND4 (39kDa), cytochrome b (35kDa), ND1 and ND2 (30kDa and 33kDa), and COXII and COXIII which co-migrate at Mf 18-20kDa (Beattie and Sen, 1987; Bodnar et al. 1995).
Figure 41. PCR products from DP-A549ρ0 cybrid clones (labelled 1-16), separated on a 1.2% agarose gel. The mtDNA specific primers have generated a 630bp mtDNA fragment from all the DP-A549ρ0 cybrid clones available for analysis.

10.6.2 MtDNA analysis of DP-A549ρ0 cybrids

Restriction enzyme digestion of a 163bp mtDNA PCR product, amplified from DNA extracted from the parental A549 cell line confirmed the presence of a known D-loop polymorphism in this cell line. The A549 cells have 4CA repeats in the region amplified, creating an Alu 1 restriction site that is not present in DNA extracted from DP which has 5CA repeats (Figure 42). This restriction site was not present in DNA extracted from DP-A549ρ0 cybrid clones 3 and 4, and the absence of this polymorphism confirms that the mtDNA in the DP-A549ρ0 cybrids was that of the donor DP and not derived from any residual A549 mtDNA that might have been present.
10.6.3 MRC function in DP-A549p0 cybrids

Four of the DP-A549p0 cybrids clones (1, 3, 4 and 15) and four from the control-A549p0 cybrids were randomly selected for MRC enzyme analysis. No statistically significant differences (Mann-Whitney U-test) in enzyme activities were observed, and the complex II/III and complex IV defects previously identified in the DP fibroblasts were no longer expressed (Table 19 and Figure 43). Statistical analysis was also performed using a student's t-test modified for use with small numbers (Parker, 1979), as had been necessary for analysis of
MRC function in the HGPS fibroblasts (Chapter 10.3). Using this test the higher CS activity in DP-A549ρ⁰ cell lines just reached significance at $p = 0.05$. This may just reflect differences in the purity of mitochondrial preparations. Although the mean complex I:CS ratio in the DP-A549ρ⁰ cybrids was 30% lower than in the control-A549ρ⁰ cybrids, this difference was not significant using either statistical test.

![Graph showing MRC enzyme activities](image)

**Figure 43.** MRC enzyme activities in MEFs from control-A549ρ⁰ (open bars) and HGPS-A549ρ⁰ (shaded bars) cybrid clones. The mean values +/- SD from three preparations from each cybrid clone analysed are shown.
**Table 19. MRC enzyme activities in MEFs from HGPS-A549ρ₀ cybrids**

A: Specific activities

<table>
<thead>
<tr>
<th></th>
<th>CS</th>
<th>NADH:CoQ₁ R</th>
<th>SCcR</th>
<th>COX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control-ρ₀ (n = 4)</td>
<td>113.1 +/- 12.5</td>
<td>48.9 +/- 9.2</td>
<td>66.2 +/- 10.8</td>
<td>2.94 +/- 0.52</td>
</tr>
<tr>
<td>DP-ρ₀ (n = 4)</td>
<td>134.6 +/- 15.6*</td>
<td>40.0 +/- 11.4</td>
<td>78.6 +/- 12.9</td>
<td>3.36 +/- 0.58</td>
</tr>
</tbody>
</table>

B: Citrate synthase ratios

<table>
<thead>
<tr>
<th></th>
<th>NADH:CoQ₁ R (X10)</th>
<th>SCcR (X10)</th>
<th>COX (X100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control-ρ₀ (n = 4)</td>
<td>4.37 +/- 0.98</td>
<td>5.88 +/- 0.68</td>
<td>2.61 +/- 0.28</td>
</tr>
<tr>
<td>DP-ρ₀ (n = 4)</td>
<td>3.06 +/- 0.99*</td>
<td>6.02 +/- 1.34</td>
<td>2.53 +/- 0.28</td>
</tr>
</tbody>
</table>

MRC function in control-A549ρ₀ cybrids and DP-A549ρ₀ cybrids. A: Specific activities are expressed as nmols/min/mg protein except for complex IV which is expressed as the first order rate constant k/min/mg protein. B: data from A expressed as a ratio with citrate synthase (CS) activity. Values are means +/- SD, calculated from the combined means of triplicate preparations from 4 separate cybrid clones derived from each cell line. Statistical analysis was performed using both the Mann-Whitney U-test, and a student's t-test modified for use with small numbers (Parker, 1979). *See text details of statistical analysis.
10.7 Discussion: mitochondria in HGPS

10.7.1 MRC function in HGPS

This is the first report demonstrating mitochondrial dysfunction in HGPS. That the defects observed can be restored to control values in the presence of a new nuclear environment, as demonstrated by the A549-ψ0 studies, implicates nuclear and not mitochondrial genes in this dysfunction.

Previous studies on energy metabolism in HGPS predated current knowledge regarding MRC enzymes. Villee et al. (1969) and Goldstein et al. (1982) studied MRC function with complex I linked substrates in cultured fibroblasts and reported normal oxygen consumption/ATP synthesis under these conditions. Thus no defect in any MRC enzyme was detected, but both groups lacked subjects with known defects as positive controls, so the sensitivity of the methods used in these studies is not known. The results presented in this thesis have demonstrated normal complex I activity, however significant defects in complex II/III and IV activities have for the first time been identified in both the HGPS patients studied here.

The controls used for this study (range 21 - 32 years) were a little older than the HGPS subjects (aged 16 and 20 years). A decline in MRC enzyme activities has been demonstrated in fibroblast cultures from ageing subjects (Chapter 9), but perfectly matched control subjects were not available. However, the fact that complex II/III and IV defects were observed in the younger HGPS patients compared to slightly older controls supports the hypothesis that true enzyme deficiencies exist. The results presented here suggest that mitochondrial dysfunction might play a role in the pathogenesis of HGPS.

The use of tissue culture models in the study of disease has its limitations, but in HGPS the skin is a major disease target clinically and was thus considered likely to demonstrate any metabolic changes related to the disease process. In addition mitochondrial dysfunction has been readily demonstrated in fibroblasts from subjects both with recognized mitochondrial
disease (Bodnar et al. 1993), and in normal ageing (Hayashi et al. 1994a). As documented in Chapter 9, this study also confirmed a significant decline in the activities of complex I and IV in cultured fibroblasts from ageing donors, demonstrating the validity of this approach.

However other factors that might influence mitochondrial function in HGPS must also be considered: the possibility that these changes are non-specific consequences of general ill health in the HGPS subjects, or secondary to medication are excluded by the use of cultured cells rather than fresh biopsy material. In both cases tissue culture conditions were identical to those used for control patients, eliminating many potential neuroendocrine or chemical influences that might be present in vivo. Another possibility is that these biochemical defects are merely a non-specific reflection of senescence in vitro. Cell division in the HG1 fibroblasts was slow at the time of harvesting for mitochondrial preparations and some cytoplasmic irregularities were visible on microscopy, both of which might be considered evidence of premature senescence. However fibroblasts from DP were not distinguishable in appearance in culture from those of controls at the time of harvest for mitochondrial preparations, showing no evidence of premature in vitro senescence. Therefore the most likely explanation for the defects seen in the HGPS patients is that mitochondrial or nuclear genes involved with the biosynthesis of the MRC are abnormal in HGPS, and that mitochondrial dysfunction may represent part of the disease process itself.

10.7.2 MtDNA in HGPS

The demonstration of the presence of mtDNA with the common deletion in HGPS cells at low levels, comparable to those of age-matched controls, indicates that the presence of mtDNA bearing this deletion is not the sole cause of mitochondrial dysfunction in HGPS. As with the results in normal ageing subjects (Chapter 9), the methods used are only semi-quantitative, and the presence of other mtDNA re-arrangements or point mutations has not been
excluded. However no single mitochondrial point mutation would be expected to produce defects in both complexes II/III and IV whilst sparing complex I. Mutations affecting mtDNA tRNA genes most commonly affect complexes I and/or IV, or all enzyme activities (Schapira, 1994a). Cytochrome b (complex III) and the mitochondrially encoded COX subunits are physically separated within the mitochondrial genome by complex I and V subunits, making it hard to envisage any mtDNA rearrangement which could selectively affect complexes II/III and IV whilst sparing complex I. Furthermore nuclear genes have been implicated in the complex IV defect seen in normal ageing (Hayashi et al. 1994a). Thus further detailed study of mtDNA in HGPS was not carried out, and the results of subsequent experiments supported a role for nuclear and not mitochondrial genes in the MRC dysfunction identified.

10.7.3 Mitochondrial proteins in HGPS

That mitochondrially encoded protein synthesis appeared normal in the in vitro translation studies suggests that mtDNA in HGPS is functionally normal. No disturbance of mtDNA translation was demonstrated. In conditions such as MERRF and MELAS abnormalities in mitochondrial protein synthesis have been readily demonstrated by similar methods to those used in this study (Koga et al. 1995; Hanna et al. 1995). Although the findings in HGPS do not exclude the presence of mitochondrial mutations, they do suggest that nuclear and not mitochondrial genes are responsible for the functional defects. In keeping with this hypothesis western blots using antibodies directed at the mitochondrially encoded COX subunits I and II detected no differences between an HGPS patient and an age matched control, despite the severe functional COX defect. Low levels of immunoreactive material using antiserum to COX subunits II and III have been reported in patients with mtDNA deletions compared to controls (Oldfors et al. 1992), and mitochondrially encoded COX subunits have been shown to be preferentially affected compared to nuclear encoded subunits in patients with the tRNA^lys mtDNA mutation by in vitro translation studies.
(Hanna et al. 1995). MtDNA mutations involving COX may also secondarily affect nuclear COX subunits, (COX IV was also low in the deletion study by Hanna et al., 1995), presumably as the assembly of the holoenzyme is disrupted. Normal levels of the nuclear encoded COX subunit IV were detected in our HGPS patient, but other sufficiently specific antibodies to nuclear COX subunits, or to complex II/III subunits, were not available. In combination with the in vitro translation findings however, no gross structural abnormalities in COX, or indeed the other mitochondrially synthesized proteins, have been demonstrated in HGPS despite the marked functional defects. Similar findings have been reported in a patient with a severe mitochondrial encephalomyopathy (Nijtmans et al. 1995), who identified a functional COX defect in cultured fibroblasts with normal synthesis, assembly and stability of both the mitochondrial and nuclear encoded COX subunits studied. Further kinetic analysis of COX in this patient suggested an aberrant interaction between COX and its substrate, probably involving a nuclear subunit, despite the fact that the mitochondrially encoded subunits are generally considered to represent the catalytic core of the enzyme (Capaldi, 1990). Of note this patient was born of consanguineous parents, supporting an autosomal recessive genetic defect, as has also been proposed in HGPS (Khalifa, 1989).

10.7.4 Nuclear complementation in HGPS

The restoration of MRC enzyme activities to levels comparable to those of a control in all of the DP-A549-\(\rho^0\) fusion clones studied supports a role for nuclear genes in the mitochondrial dysfunction identified in the HGPS fibroblasts. None the less, as was the case in the LHON fusion studies (Chapter 7) other explanations must be considered.

Firstly, as the A549-\(\rho^0\) cells are lung derived in contrast to the fibroblasts, the correction of MRC activities in the DP-A549\(\rho^0\) cells might represent the influence of normal tissue-specific factors rather than the absence of putative pathogenetic nuclear mutation(s). However COX defects
have been seen to persist in the A549 nuclear environment associated with the 3243tRNA^Leu(UUR) mutation (Dunbar et al. 1995; 1996), implying that a tissue-type change alone is insufficient to correct the defect, and mitochondrially determined MRC defects can persist in this particular nuclear background.

Secondly a mtDNA mutation in HGPS cells has not been definitely excluded. If present it may be so in a heteroplasmic state as is the case in most mitochondrial diseases, with the proportion of mutant and wild-type mtDNA varying between individual cells. It is conceivable that by chance only those with low mutant loads have fused to make the DP-A549ρ^0 cybrids subsequently analysed, or that the new A549 nuclear environment might have influenced genotype drift towards wild-type mtDNA in the cybrids. Dunbar et al. (1995) did find that the A549 nucleus seemed to select for wild-type mtDNA in heteroplasmic 3243- A549ρ^0 cybrids in culture. In the case of DP this could account for the results obtained where a pathogenetic mitochondrial mutation did exist in the fibroblasts.

Finally due to the limited sample supply and concerns over the in vitro life span of those HGPS cells available, growth curves in both supplemented growth medium and selection medium were not performed. Thus it is not known whether or not the selection medium, lacking pyruvate and uridine, preferentially selected against HGPS cells potentially carrying mtDNA abnormalities and expressing biochemical defects. Although mtDNA analysis was able to confirm that mtDNA in the DP-A549ρ^0 cell was that of DP, as no putative pathogenetic mtDNA mutation was identified it was not possible to ascertain the presence of absence of such a mutation in growing cybrids.

The proposed role of nuclear genes in mitochondrial dysfunction in HGPS might be confirmed by a mirror fusion experiment in which mitochondria from control fibroblasts are introduced in to an HGPS cell which has itself been rendered ρ^0. However this would require transformation of the HGPS cells in order to extend their replicative capacity which was felt to be inappropriate. As senescence is a characteristic of major interest in HGPS, transformation might
eliminate expression of genes important to the HGPS phenotype. This has been observed in other studies where pre-existing abnormalities in DNA-repair were no longer expressed in transformed HGPS fibroblasts (Saito and Moses, 1991).

Whilst further work is required, the most likely explanation for restored MRC function in the DP A549ρ0 cybrids, is that nuclear genes play a key role in mitochondrial dysfunction in HGPS. The possible nature of these nuclear genes and the role of mitochondrial dysfunction in the context of the clinical features of HGPS will be discussed further in Chapter 11.
Chapter 11. Mitochondrial dysfunction in the pathogenesis of HGPS

11.1 Final discussion: mitochondrial dysfunction in the pathogenesis of HGPS

The data in this study demonstrates abnormal mitochondrial function in cell cultures from an affected tissue in HGPS. The decline in MRC activity in HGPS is similar, though not identical, to that seen in biological ageing with which HGPS shares some clinical features. Nuclear genomic transplantation experiments in addition to structural and functional assessments of mtDNA in HGPS fibroblasts suggest that the cause(s) of the mitochondrial abnormalities demonstrated in this disorder resides in the nucleus. These results, together with those of Hayashi et al. (1994) would also support the view that mitochondrial dysfunction in biological ageing is primarily due to nuclear genomic defects.

The extent to which this mitochondrial dysfunction is itself involved in disease pathogenesis in HGPS, rather than representing changes secondary to other processes must next be considered. It is well supported that disturbances in mitochondrial function are of primary pathogenetic significance in the many established mitochondrial diseases where specific mutations have been identified, both of nuclear and of mitochondrial genes, as discussed in Chapter 1.3. However even in these conditions the mechanisms that lead to the final clinical presentation are not well understood. When considering biological ageing and neurodegenerative diseases the role of mitochondria in pathogenesis is even more unclear. As an example, although for a complex I defect identified in Parkinson's disease substantia nigra there is much evidence linking the biochemical defect and the clinical/pathological disease entity, a primary aetiological role for complex I dysfunction is far from proven (Tipton and Singer, 1993; Schapira, 1994b). Numerous other factors such as oxidative defences, free radical production, metal concentrations and exogenous toxins are also candidates in a complicated cycle of events. The same reasoning must be applied to the MRC dysfunction identified in HGPS patients and in ageing. For example abnormalities in oxidative defences might be primarily affected in
HGPS, and MRC dysfunction a secondary phenomenon. None the less MRC
dysfunction could contribute to the clinical phenotype as a final common
pathway resulting in a premature decline in ATP dependant cell functions, and
ultimately in cell death.

If it is accepted that nuclearly determined mitochondrial dysfunction
occurs in HGPS, the possible identity of the responsible nuclear genes must
then be considered. This work has shown both complexes II/III and IV to be
affected. An abnormal nuclear gene encoding MRC proteins is therefore
unlikely, as only a single enzyme defect would be anticipated as a result of
such a mutation. A nuclear mutation in a protein involved in MRC biogenesis is
a further possibility, although it would again have to preferentially affect a
process common to the affected enzyme complexes, yet sparing complex I.
Although our understanding of mitochondrial protein import mechanisms, and of
MRC biogenesis is expanding, few nuclear genes involved in these processes
have yet been characterized, and certainly no nuclear gene that would account
for the defects found in HGPS has yet been identified. In proposing candidate
genes those involved in cytochrome biosynthesis might be specifically
considered, as the enzyme assays for complex II/III and IV both involve
cytochromes as electron transfer intermediaries, albeit in different forms.
Further work to define the cytochrome content of HGPS mitochondria might
take this further. However although such avenues should be addressed, it is
possible that previously identified abnormalities in HGPS such as defective
nuclear DNA repair mechanisms (Wang et al. 1991) or shortened telomeres
(Allsopp et al. 1992), represent the primary abnormality, with a secondary effect
on mitochondrial function through damaged nuclear genes involved in MRC
biogenesis or function. The recent identification of a mutated putative DNA
helicase as the gene product of the Werner's syndrome gene, a less severe
progeroid syndrome, is of particular interest in this respect (Yu et al. 1996). In
this scenario MRC dysfunction could still play a role in disease pathogenesis,
albeit a secondary one, and contribute to the clinical phenotype. Detailed
studies of mitochondrial function have not been reported in Werner's syndrome, but would be important to study this hypothesis further.

If MRC dysfunction is important in HGPS, how might it lead to the clinical features commonly associated? This study has only considered fibroblast mitochondria, and clearly a study of other tissues would be important. Is there evidence of MRC dysfunction in the cardiovascular tissues of HGPS subjects, also a major disease target clinically? MRC dysfunction is linked with cardiovascular disease in other mitochondrial disease models: both cardiac muscle abnormalities and conduction defects are not infrequent in mitochondrial disease such as MELAS, MERRF and Kearns-Sayre syndrome (Anan et al. 1995), and the number of COX deficient cardiomyocytes has been reported to increase with physiological ageing (Müller-Höcker, 1989), commonly accompanied by an increase in cardiac disease. Mitochondrial dysfunction may also have indirect effects on vasculature through free radical production which is increased in the presence of mitochondrial defects (Turrens and Boveris, 1980). Markers of free radical damage have been reported as age related phenomena in rat cerebral microvessels (Mooradian and Uko-eninn, 1995), and damage to larger vessels with consequent premature atherosclerosis is also feasible. A high incidence of vascular disease is not then surprising in a model of HGPS involving MRC dysfunction as of clinical significance. The lack of neurodegenerative changes in HGPS is perhaps harder to account for, given the abundance of evidence linking MRC dysfunction to age related neurodegenerative diseases (Ozawa, 1995; Flint-Beal, 1992). However such discrepancies have almost become the hallmark of mitochondrial diseases in recent years, and clearly there is a long way to go in understanding the link between molecular and clinical phenotypes. Even in conditions where the molecular defect has been well characterized this relationship is not well understood. Given the preliminary nature of the data presented here on mitochondrial function in HGPS, any comment on the role of mitochondria in
the pathogenesis of HGPS must at best be speculative until further studies have been performed.

11.2 Future work in the study of HGPS

The data in this thesis was taken from only two HGPS patients, and some of the studies were confined to fibroblasts from a single patient. This reflects the rarity of the condition, and difficulty maintaining cells from HGPS subjects in culture. The study of further tissues from these patients, and on a greater number of subjects are desirable in order to extend the data. This should include other methodologies for assessing mitochondrial function including polarography, ATP synthesis, and \textit{in vivo} approaches such as MR Spectroscopy. In addition post mortem studies on HGPS patients looking specifically for histological features associated with mitochondrial disease would be of interest.

Further studies should also be performed addressing the contribution of nuclear genes to mitochondrial dysfunction in HGPS. Firstly the remaining DP-A549\(\rho^0\) fusion clones should be analysed to establish whether normal MRC function has been restored to all, or whether any retain the defects. The latter case would suggest the presence of a mitochondrial mutation(s) responsible for the enzyme defects, which have not been maintained in the corrected fusion clones. As previously discussed (Chapter 10.7.4) this is a possible explanation for the apparent restoration of enzyme activities in DP-A549\(\rho^0\) fusion clones, as a mtDNA mutation was not definitively excluded from DP cells. Normal MRC function in the presence of other nuclear environments by fusion of HGPS cytoplasts with different \(\rho^0\) cell types, ideally \(\rho^0\) fibroblasts, would exclude the possibility that restoration of normal MRC function merely reflected a tissue type change, or was a feature peculiar to the A549 cells. In addition fusion of non-enucleated HGPS cells with \(\rho^0\) cells would address whether dominant or recessive nuclear genes in HGPS cells were responsible for the MRC dysfunction. If dominant fusion cybrids containing nuclear material from both
the $\rho^0$ cells and the original HGPS cells would still express the defects, whereas the presence of non-HGPS nuclear material from $\rho^0$ cells would be able to restore normal MRC function to such fusions if the still present defective HGPS genes were recessive. Further studies could then be undertaken to localize the defective gene(s) by microcell fusions and karyotyping (Lugo et al. 1987).

Although the data suggests that nuclear and not mitochondrial genes are important to the MRC defects identified in HGPS, it is not absolutely conclusive and therefore further study of mtDNA in HGPS cells is indicated. In particular quantitation of levels of mtDNA with the common deletion in tissues from HGPS subjects, rather than in culture, would be of interest. Levels in culture may not reflect those in vivo. Were tissues from HGPS subjects to have significantly increased levels of deleted mtDNA compared to age-matched controls, this would support similarities between HGPS and physiological ageing in which an age-related accumulation of deleted mtDNA is well documented (Baumer et al. 1994). Whether such an accumulation of mutated mtDNA in ageing represents the cause of MRC dysfunction, or is consequent upon it remains to be seen, although the failure to demonstrate an excess of deleted mtDNA in patients with other mitochondrial diseases such as MELAS and MERRF (Moraes et al. 1995) argues against mitochondrial dysfunction resulting in the accumulation of deleted mtDNA.

Finally, given the relationship between mitochondria, free radicals and oxidative defences, and the implications of all in physiological ageing (Shigenaga et al. 1994), HGPS tissues should be assessed for abnormalities in oxidative defences, and for markers of oxidative damage. Many techniques are now well established in this field, and the limitation for such work would again be the supply of tissues and subjects with HGPS, rather than in methodological difficulties. Unfortunately this factor is largely insurmountable, and will undoubtedly continue to hamper study of this fascinating, albeit rare condition.
References


Hutchinson J. (1886) Congenital absence of hair and mammary glands with atrophic condition of the skin and its appendages in a boy whose mother had been almost totally bald from alopecia areata from the age of six. Med. Chirurg. Trans. 69, 36


Appendices

A 3,460 LHON family tree

Subjects studied are indicated by their conventional numbers.

B 11,778 LHON family tree

Subjects studied are indicated by their conventional numbers.
Subjects studied are indicated by their conventional numbers. Subject III_8 died 2 years after fibroblast samples were obtained.