PCR analysis of the mouse mitochondrial genome to assess whether deletions accumulate with age

Louise Anne Harkin

A thesis submitted for the degree of
Master of Science
in
The University of Adelaide
(Faculty of Science)
December 1994

Awarded 1995

For my Family

Table of Contents:

Declaration (i)
Acknowledgments (ii)
Summary (iii)

Chapter 1: Literature review

1.1.0. Mitochondria and their own genome:- 1

1.1.1. Mitochondrial oxidative phosphorylation 3

1.1.2. The mammalian mitochondrial genome 5

1.1.3. Susceptibility of the mitochondrial genome to mutations 8
(i) MtDNA is a naked molecule with a high informational density 9
(ii) MtDNA is replicated with low fidelity 9
(iii) MtDNA is continuously exposed to a flux of reactive oxygen species 10
(iv) MtDNA is sensitive to various chemicals 13
(v) Lack of repair mechanisms within the mitochondria 14

1.1.4. Mutations of the mitochondrial genome 15

1.2.0. MtDNA mutations in association with mitochondrial diseases:- 17

1.2.1. Disease related mtDNA nucleotide substitutions 18
(i) Missense mutations which alter electron transport proteins 18
(ii) Missense mutations which effect ATPase Synthase 22
(iii) Base substitutions which effect mtDNA protein synthesis 23

1.2.2. Disease related mtDNA deletions 25

1.2.3. Direct repeat sequences associated with mtDNA deletions 30

1.2.4. Deleted mtDNA and cytochrome c oxidase deficiency 31

1.2.5. MtDNA mutations associated with diabetes mellitus 31

1.2.6. MtDNA mutations associated with degenerative diseases 32

1.3.0. Mitochondrial DNA alterations as ageing associated molecular events:- 34

1.3.1. Free radical theory of ageing 35

1.3.2. Changes in mtDNA structure and transcription with age 37

1.3.3. Age-related mtDNA base substitutions 39

1.3.4. Age-related deletions within the human mitochondrial genome 41

1.3.5. Quantitation of the level of deleted mtDNA molecules in ageing human tissues 53

1.3.6. Age-associated mtDNA deletions in other species 58

1.3.7. Theories on the mechanism of deletion formation in mitochondrial DNA 59

1.3.8. Consequences of the accumulation of deleted mtDNA molecules with age 65

1.4.0. Objectives of this study:- 68

Chapter 2: Materials and Methods

2.0. Experimental animals:- 70

2.1. Mouse mitochondrial DNA isolation / purification / quantitation:- 71

2.1.1. Isolation of mitochondria 71
2.1.2. Mitochondrial DNA isolation 71
2.1.3. Purification of mitochondrial DNA
(i) Phenol / chloroform / iso-amyl alcohol extraction 72
(ii) Prep-A-Gene purification matrix kit (BIORAD®) 73
2.1.4. Quantitation of mitochondrial DNA 74

2.2. Polymerase Chain Reaction (PCR) analysis:- 74

2.2.1. PCR reaction reagents 74
2.2.2. Mouse mitochondrial DNA PCR primers 75
2.2.3. PCR thermal cycling conditions 75
2.2.4. Hot start PCR method 75
2.2.5. Agarose gel electrophoresis 76

2.3. Molecular engineering of mouse mitochondrial DNA deletions:- 77

2.3.1. Computer analysis 77
2.3.2. Nhe 1 restriction endonuclease digestion 77
2.3.3. T4 DNA ligase ligation reaction 77

2.4. Mapping of direct repeat sequences within the mouse mitochondrial genome:- 78

2.5. Buffers and reagents:- 78

2.6. Chemicals:- 81

Chapter 3: Development of a PCR method for detecting deletions within the mouse mitochondrial genome

3.0. Introduction:- 86

3.1. Design of mouse mitochondrial DNA primers:- 87

Experimental Design:- 87

Results:- 88

Summary:- 91

3.2. Optimization of the polymerase Chain Reaction conditions:- 93

Experimental design:- 93

Results:-

3.2.1. Optimization of the Taq DNA polymerase and $MgCl_2$ concentrations 94
3.2.2. Optimization of the mitochondrial DNA template concentration 94
3.2.3. Optimization of the concentration of each primer 96
3.2.4. Optimization of the concentration of each dNTP 96
3.2.5. Optimization of the PCR extension time 98
3.2.6. Optimization of the PCR denaturation time 98
3.2.7. Optimization of the PCR annealing time 98
3.2.8. Optimization of the PCR extension temperature 101
3.2.9. Optimization of the PCR denaturation temperature 101
3.2.10. Optimization of the PCR annealing temperature 101
3.2.11. Analysis of all primers using the optimized PCR conditions 101

Summary:- 104

3.3. Design of a PCR method for detecting deletions within the mouse mitochondrial genome:- 106

Experimental design:- 106

Results:- 109

Summary:- 113

3.4. PCR analysis of genetically engineered deleted mitochondrial DNA molecules:- 113

Experimental design:- 113

Results:-
3.4.1. Selection of a restriction endonuclease to produce deleted mtDNA molecules 116
3.4.2. Genetic engineering of deleted mtDNA molecules 119
3.4.3. PCR analysis of genetically engineered deleted mtDNA molecules 119
3.4.4. Estimation of the ratio of deleted mtDNA : wildtype mtDNA 123
3.4.5. Estimation of the lowest % deleted mtDNA detectable by the PCR method 123

Summary:- 126

3.5. Conclusions:- 126

Chapter 4: PCR analysis of mouse mitochondrial DNA for the presence of age-related deletions

4.0. Introduction:- 128

4.1. Analysis of various aged mice tissues by Part A of the developed PCR method:- 129

Experimental design:- 129

Results:-
4.1.1. Analysis of brain mtDNA by part A of the developed PCR method 131
4.1.2. Analysis of heart mtDNA by part A of the developed PCR method 135
4.1.3. Analysis of skeletal muscle mtDNA by part A of the developed PCR method 139
4.1.4. Analysis of liver mtDNA by part A of the developed PCR method 143

Summary:- 147

4.2. Analysis of mtDNA from aged mice brains by part B of the developed PCR method:- 148

Experimental design:- 148

Results:- 149

Summary:- 157

4.3. The effect of reducing the PCR extension time on the part A analysis of different aged brain mtDNA samples:- 158

Experimental design:- 158

Results:- 158

Summary:- 162

4.4. The effect of increasing the number of PCR cycles on the part A PCR analysis of different aged brain mtDNA samples:- 162

Experimental design:- 162

Results:- 163

Summary:- 165

4.5. Conclusions:- 165

Chapter 5: Direct repeat sequences within the mouse mitochondrial genome

5.0. Introduction:- 167

5.1. Direct repeat sequences within the mouse mitochondrial genome:- 168

Experimental design:- 168

Results:- 168

Summary:- 177

5.2. PCR analysis of brain mtDNA from the nucleotide position 8211 to 79:- 178

Experimental design:- 178

Results:-

5.2.1. PCR analysis of different aged brain mtDNA samples 180

5.2.2. PCR analysis of different aged brain mtDNA samples using reduced PCR extension times 182

5.2.3. PCR analysis of different aged brain mtDNA samples for a total of 80 PCR cycles 186

Summary:- 188

5.3. Conclusions:- 188

Chapter 6: General discussion

6.0. Introduction:- 190

6.1. Development of a PCR method for detecting deletions within the mouse mitochondrial genome:- 191

6.2. Analysis of aged mouse mtDNA for the presence of deletions:- 193

6.3. Analysis of direct repeats sequences within the mouse mitochondrial genome:- 195

6.4. Reasons why deletions were not detected within the ageing mouse mitochondrial genome:- 196

6.5. Conclusions and future directions:- 202

Appendix 207

Bibliography 208

Declaration:

I declare this thesis to be on original material obtained while I was enrolled as a Master of Science student in the Department of Clinical and Experimental Pharmacology at The University of Adelaide. To the best of my knowledge and belief, this work does not contain whole or part of any previous studies conducted at this or any other University, except where due reference is cited.

I give consent to my thesis, when deposited within the University Library, being available for loan and photocopying.

20·12·94

Acknowledgments:

I would like to extend my sincere thanks and gratitude to Dr Michael Fenech, Dr Ivor Dreosti and Prof. Richard Head for supervising the work presented within this thesis and for providing generous guidance and constant encouragement throughout the course of the degree. I am also grateful for their time, discussions and helpful knowledge given in proof reading this thesis during its preparation.

Many thanks to all the staff at CSIRO-Division of Human Nutrition for their support and encouragement. In particular I would like to thank everyone within program H for the interest and support they have given and for providing a friendly environment in which to work.

I am also grateful to the photographic unit at the Women's and Children's Hospital (Foundation Studios) for printing the photographs presented within this thesis.

Finally my most sincere thanks go to my parents Marie and Denis, and to my family Claire, Miriam, Damien and Blazenka for their unfailing love, endless support and continuous encouragement.

Special thanks are also extended to the CSIRO-Division of Human Nutrition for providing a Postgraduate Research Scholarship, without which this project would not have been possible.

Summary:

It has been hypothesized that the accumulation of somatic gene mutations in the mitochondrial DNA (mtDNA) genome during life, and the consequential changes in cellular energetics, may contribute significantly to the ageing process. Recent evidence, utilizing PCR analysis has shown the age-related occurrence of multiple deletions of the mitochondrial genome in various human, non-proliferative tissues, where the majority of these deletions lie within the confines of tandem direct repeat sequences. These deletions have been found to arise spontaneously in the mitochondrial genome during the normal lifespan of a human individual, where they co-exist with the wildtype mtDNA population in a situation known as heteroplasmy. Quantitative analysis has shown that the level of these deleted genomes increases exponentially with age reaching levels of greater than 0.01% of the total mtDNA population.

The overall aim of this study was to determine if the accumulation of deleted mtDNA molecules in tissues during the normal ageing process is confined to the human, or whether it is a phenomenon also shared by other species. The ageing mouse was selected as an experimental model, where the brain, heart, skeletal muscle and liver were analyzed from 10 week old, 19 month old and 42 month old female *Balb C* mice for the presence of deleted mtDNA molecules. Due to the unknown size, number and location of deletions which may be present within the ageing mouse genome, a PCR method was developed, which involved using multiple primer pair combinations to methodically scan the major region of the genome between the two origins of replication, since this major arc has been found to harbor the majority of the human age-associated deletions. This PCR method relied upon relatively large deletions in the mitochondrial genome to bring two primers, that lie outside of the deletion boundaries, close enough together to efficiently amplify a deleted PCR product.

The ability of this PCR method to amplify deleted PCR products, was verified by analyzing a mixture of mitochondrial DNA which contained both the wildtype mouse mtDNA genome and deleted mtDNA molecules that were produced by genetic engineering. The designed PCR method was found to be successful in amplifying PCR products from either the wildtype or the deleted templates where the products displayed corresponding shifts in sizes when the position of either primer was independently shifted. The lowest percentage of deleted mtDNA molecules which still resulted in a detectable deleted PCR product was approximately 0.01%. This indicated that the PCR method was capable of detecting the presence of 1 deleted mtDNA molecule amongst every 10 000 wildtype genomes.

Using this designed PCR method, no age dependent or tissue specific deleted PCR products were amplified from the mtDNA samples isolated from the various aged mice. Overall, the same size products were generated by each primer pair regardless of the age or tissue examined. The products amplified by each primer pair also failed to display a corresponding shift in size when either of the primers were independently located further apart, which suggests that these DNA fragments were not genuine PCR products but were instead most likely artifacts of the PCR technique. In an attempt to enhance the amplification of the smaller and rarer deleted PCR products, the PCR experiments were repeated where the extension time was decreased and the number of PCR cycles was increased. However, both additional experiments failed to generate any genuine deleted PCR products.

Since the majority of deletions within the mitochondrial genomes of diseased and normally ageing humans involve the presence of direct repeat sequences, tandem direct repeats within the mouse mitochondrial genome were also analyzed in an effort to predict the location of deletions in the ageing mouse. Following the examination of all the repeats which were 10bp or greater, only two of these were found to share the same gene locations as the 13bp repeat sequences which surround the most common 4977bp deletion in the human genome, and the greatest percent homology either of these shared with the 13bp repeat was less than 50%. Modifying the previous PCR method to allow

amplification of the regions spanned by all the direct repeats of 10bp or greater failed to generate any deleted PCR products. Again, decreasing the extension time and increasing the number of PCR amplification cycles did not enhance the synthesis of any deleted PCR products displaying a primer shift pattern.

In conclusion, this study did not find a positive relationship between the accumulation of deleted mtDNA molecules and the ageing process of the mouse. No age dependent or tissue specific deleted PCR products were amplified by any of the primer pair combinations utilized within this entire study. Therefore, this negative result suggests that mtDNA molecules bearing deletions in the major region between the replication origins of the mitochondrial genome do not accumulate within the brain, heart, skeletal muscle and liver of ageing mice. While this result is not supportive of the view shared by other researchers, that mitochondrial DNA deletions are associated with the ageing process, it does not exclude the possibility that other mutations of the mitochondrial genome (including deletions in other regions of the mtDNA) are important contributory factors to the ageing process.