PCR analysis

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

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For my Family

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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.

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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.

Chapter 1:

Literature Review

1.1.0. Mitochondria and their own genome:-

Mitochondria are oval shaped, cytoplasmic organelles which are primarily concerned with generating energy within aerobic cells. Electron microscopic studies by Palade and Sjostrand (Stryer 1981) revealed that these organelles are composed of a two membrane system, which includes an outer smooth membrane and an inner, highly convoluted membrane. As a result, these membranes result in the formation of two separate compartments; 1) the intermembrane space between the two membranes, and 2) the matrix which is entirely bounded by the inner membrane (see Diagram 1.1). Experiments by Nass and coworkers subsequently revealed that each mitochondrion contains its own genome which is physically and genetically distinct from the nuclear DNA (reviewed by Nass 1969), known as mitochondrial DNA (mtDNA). In contrast to the nucleus, which only contains one nuclear genome, recent studies have shown that the matrix of each of these energy synthesizing organelles, contains multiple copies of the mtDNA genome, which can range from 2 to 10 genomes per mitochondrion (Robin & Wong 1988, Anderson et al 1981, Bogenhagen & Clayton 1974, Shay et al 1990). Therefore, due to the vast number of mitochondria within the cellular cytoplasm, which can range from a few in resting lymphocytes (Iwama & Eguchi 1986), to over 1000 mitochondria in hepatocytes (Flickinger et al 1979), the mammalian cell can contain several thousand copies of this species specific, closed circular mitochondrial genome.

Since the primary function of each mitochondrion is to generate cellular energy in the form of a special energy carrier known as adenosine triphosphate (ATP), it was

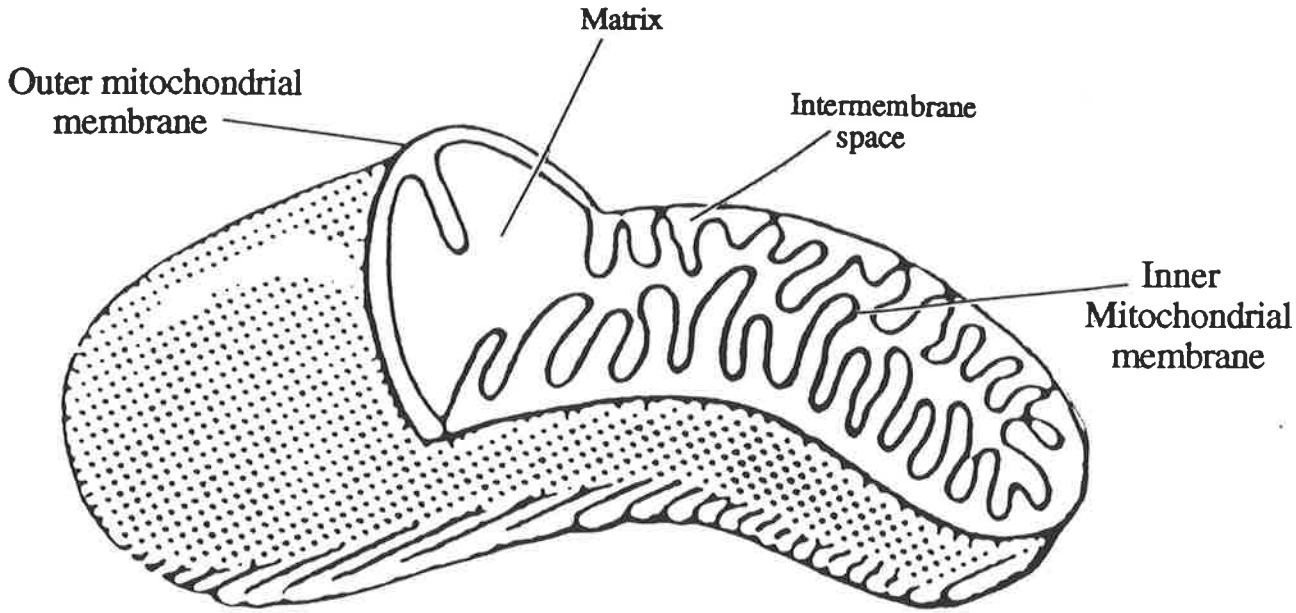


Diagram 1.1. Structure of Mitochondria:-
This diagram displays a cross-sectional view of a typical mammalian mitochondrion, which is composed of a two membrane system which includes an outer, smooth membrane and an inner, extensively folded membrane. The structure of these two membranes results in the formation of two separate compartments; the intermembrane space between the two membranes, and the matrix which is entirely surrounded by the inner membrane. The mitochondrial DNA genome is confined to the matrix of these energy synthesizing organelles.

not suprising, following the sequencing of mitochondrial DNA (Anderson et al 1981), to find that this genome encoded for several subunits of the mitochondrial oxidative phosphorylation system, which is the final, most critical pathway resulting in the generation of cellular ATP within aerobic cells.

1.1.1. Mitochondrial Oxidative Phosphorylation:-

The oxidative phosphorylation system (OXPHOS) generates mitochondrial ATP by means of five, multiple subunit, respiratory enzyme complexes which are confined to the inner mitochondrial membrane (see Diagram 1.2). Complexes I to IV form the electron transport chain, where electrons from NADH and $FADH_2$ are transferred to molecular oxygen via a series of electron carriers. The energy released during this electron flow process is used to pump protons out of the mitochondrial matrix through complexes I, III and IV. The resulting electrochemical gradient is then utilized by complex V (ATP synthase) as a source of energy, which contains a proton channel and allows the protons to return to the matrix of the mitochondrion, resulting in ADP and Pi (inorganic phosphate) condensing to form ATP. This system is the main source of ATP for several organs and tissues including the brain, muscle, heart, kidney and liver, where the rate of OXPHOS is determined by the cellular need for ATP.

The genetics of this OXPHOS process are complex in that all of these respiratory chain enzymes, except complex II, are the products of two separate genomes, where the mitochondrial DNA encodes for 13 subunits of these respiratory enzyme complexes (Anderson et al 1981), while the remaining 54 subunits are biosynthesized from information encoded in the nuclear genome. Complex I (NADH dehydrogenase) is composed of a total of 30 polypeptides of which 7 are encoded by the mitochondrial genome, in contrast to complex II (Succinate dehydrogenase) where all four peptides are encoded on the nuclear genome. There are a total of 10 polypeptides within complex III (Ubiquinol-cytochrome c oxidoreductase), where one

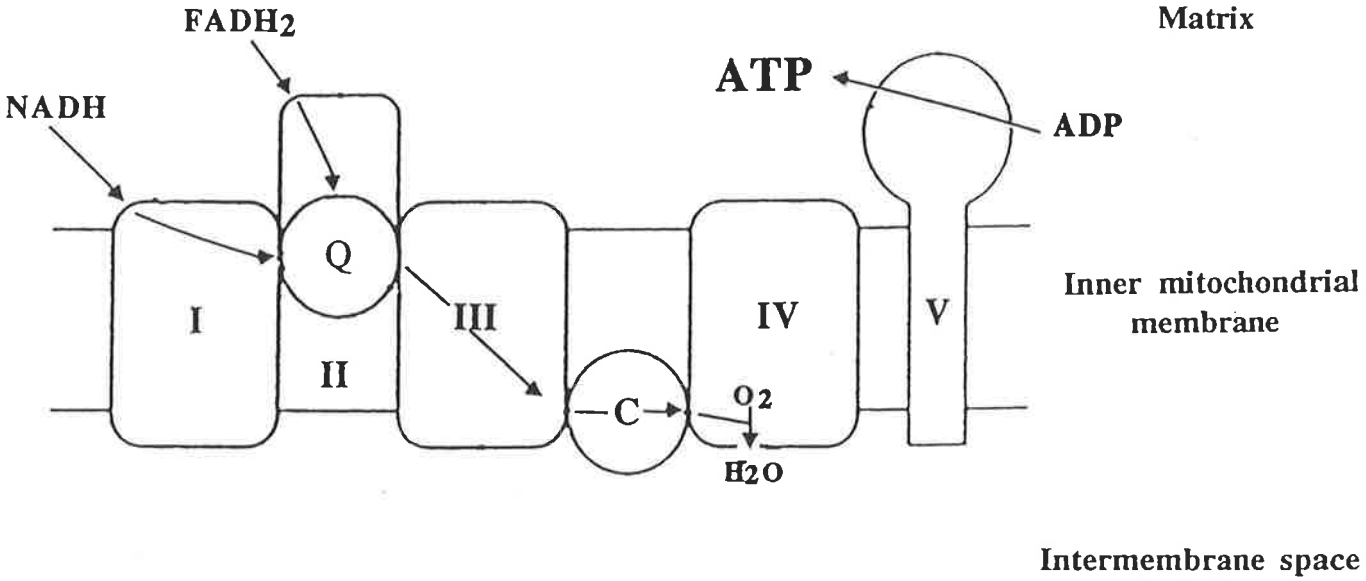


Diagram 1.2. The mitochondrial oxidative phosphorylation system:-
Mitochondrial oxidative phosphorylation involves five respiratory enzyme complexes which are located within the inner mitochondrial membrane. These complexes include; complex I (NADH dehydrogenase), complex II (Succinate dehydrogenase), complex III (Ubiquinol-cytochrome c oxidoreductase), complex IV (Cytochrome c oxidase) and complex V (ATPase synthase). Complexes I to IV form the electron transport chain, where electrons from NADH and $FADH_2$ are transferred to molecular oxygen via series of electron carriers. The energy released during this electron flow process results in the pumping of protons out of the matrix and into the intermembrane space through complexes I, III, and IV. The resulting electrochemical gradient is then utilized by complex V, which contains a proton channel and allows the protons to return to the matrix resulting in the phosphorylation of ADP to yield ATP.

of these is dependent upon the expression of the mitochondrial genome. Complex IV (Cytochrome c oxidase) is composed of 13 subunits of which 3 are encoded in the mitochondrial genome, while this cytoplasmic genome also encodes for 2 subunits of complex V (ATP synthase).

Those subunits which are encoded by the mtDNA are transcribed and translated on mitochondrial ribosomes within the mitochondria (Anderson et al 1981), whereas the nuclear encoded subunits are translated by the cytoplasmic protein synthetic apparatus with, in the majority of cases, N-terminal pre-sequences that target protein receptor sites on the outer mitochondrial membrane. The immature protein is then transported across the mitochondrial membranes, the targeting sequence is removed and the mature protein is inserted into the haloenzyme alongside the mitochondrially encoded proteins (Anderson et al 1981, Tzagoloff 1982, Tzagoloff & Myers 1986).

1.1.2. The mammalian mitochondrial genome:-

Mitochondrial DNA (mtDNA) is a small circular doubled-stranded molecule of 16.5 kilobases (Kb) long, whose gene expression is essential for mitochondrial respiratory function (Anderson et al 1981) (see Diagram 1.3). The majority of these molecules exist in the basic monomeric form however, most mammalian cells also maintain a significant proportion of their mtDNA in the form of catenated circles, in which monomer units are joined as links in a chain (Clayton 1982). In addition to encoding for 13 subunits of the oxidative phosphorylation system which include, seven subunits of complex I; ND1, ND2, ND3, ND4L, ND4, ND5 and ND6, one subunit of complex III; cytochrome b, three subunits of complex IV; COI, COII and COIII, and two subunits of complex V; ATPase 6 and ATPase 8, this genome also contains the sequences for two ribosomal RNA (rRNA) and 22 transfer RNA (tRNA) molecules which are required for the mRNA translation of the 13 protein coding genes (Anderson et al 1981).

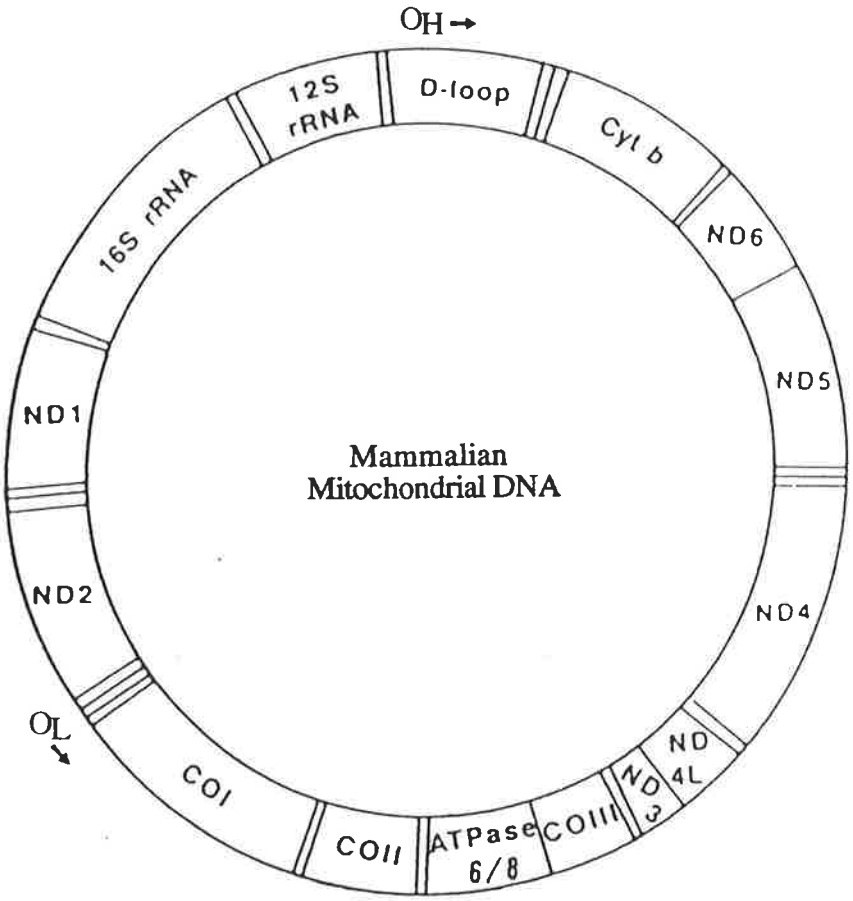


Diagram 1.3. Genetic map of the mammalian mitochondrial genome:-
This diagram displays the spatial location of all the genes within the mammalian mitochondrial genome, which are responsible for encoding for 13 key subunits of the oxidative phosphorylation respiratory enzyme complexes I - V. The open bars represent transfer RNA (tRNA) genes; 12SrRNA and 16SrRNA are ribosomal RNA genes; ND1, ND2, ND3, ND4L, ND4, ND5 and ND6 genes, encode for subunits 1, 2, 3, 4L, 4, 5 and 6 of NADH dehydrogenase (complex I); COI, COII and COIII genes encode for subunits 1, 2 and 3 of cytochrome c oxidase (complex IV); cyt. b gene, encodes for cytochrome b which is a subunit of Ubiquinol-cytochrome c oxidoreductase (complex III), and ATPase 6/8 genes encode for subunits 6 and 8 of ATPase synthase (complex V). The light and heavy strand origins of replication are denoted as O_L and O_H respectively.

The major form of mtDNA in mammalian cells is a covalently closed circle which has two distinct replication origins (O_H and O_L), with a displacement loop (D loop) at the origin of replication of the heavy (H) strand. This triplex D loop structure is formed by the synthesis of a short daughter H strand of approximately 680 bases, which remains stably associated with the parental closed circle. The length of this D-loop H strand has been found to vary, existing as a family of discrete lengths (Gillum & Clayton 1978).

Replication of the mitochondrial genome occurs independently of the cell cycle and independently of mitochondrion division (Bogenhagen & Clayton 1977, Birky 1983). Initiation of the heavy (H) strand synthesis proceeds at a specific origin within the D loop region (Crews et al 1979, Gillum & Clayton 1979), and continues in a unidirectional, clockwise manner until completion. When H-strand synthesis is two thirds complete, the origin of synthesis of the light (L) strand (Martens & Clayton 1979, Berk & Clayton 1974), is exposed as a single stranded template and initiation of L-strand synthesis begins to occur unidirectionally, but in the counterclock wise direction. Thus, the replication of this genome is conserved where each daughter genome contains one parental strand and one newly synthesized strand (reviewed by Clayton 1982).

Transcription of mammalian mtDNA is also unique in that both strands seem to be completely transcribed from promoters (PL and PH1) situated in the D loop region (Aloni & Attardi 1971, Murphy et al 1975). These primary transcripts are then processed to give the 12S and 16S rRNAs, tRNAs and a number of mRNAs, which are not capped but are polyadenylated (Ojala et al 1981, Montoya et al 1981). The major coding strand of the mitochondrial genome is the heavy (H) strand, which encodes all of the RNAs and protein products except for the ND6 protein and 14 tRNA which are specified by the L strand (Grivell 1989).

In contrast to the nuclear genome, the inheritance of mtDNA is almost purely maternal, where the mtDNA is transmitted exclusively by mothers through the egg cytoplasm to all of their off spring, and their daughters subsequently pass it on to their

children (Case & Wallace 1981, Giles et al 1980), however, a small paternal contribution has been identified in mice (Gyllensten et al 1991). Another striking feature of this genome is its extreme economy (Borst & Grivell 1981). Nearly every base pair in the mtDNA contributes to encoding for a protein or an RNA product, and some even have a double role in that the last base of one gene serves as the first one of the next gene. Only two small segments of the genome, the displacement loop (D-loop) region containing the origin of heavy strand replication and the origin of light strand replication, do not contribute to the code of a functional RNA species.

In addition, the genetic code of the mitochondrial genome varies slightly from the universal code (Anderson et al 1981). It is read in a unique fashion by only 22 tRNA which are all encoded by the mitochondrial genome, in contrast to 32 tRNA which is the minimum number of tRNAs required to translate the classic genetic code. No tRNA genes other than the 22 previously mentioned can be found in the mammalian mitochondrial genome, and no mammalian tRNAs appear to be imported from the cytoplasm to function in mitochondrial translation (Aujame & Freeman 1979). A mechanism by which the 22 human mitochondrial tRNAs are sufficient to translate all internal codons of mitochondrial reading frames has been proposed by Barrell et al (1980), where more than one codon is read by a single tRNA. For example, GUU, GUC, GUA, and GUG are all read by $tRNA^{val}$.

Animal mtDNA is also unusually susceptible to strand cleavage at high pH, and exposure to RNAase, which has been found to be due to the presence of ribonucleotides in closed circular mtDNA. Direct evidence for the presence of these RNA bases in mouse mtDNA was obtained by Brennicke & Clayton (1981), where the major sites of ribosubstitution were observed to be in the two replication origin regions.

1.1.3. Susceptibility of the mitochondrial genome to mutations:-

Energy supply is of primary importance in maintaining the quality of cell function. Apart from a minor contribution of anaerobic glycolysis, oxidative

phosphorylation is exclusively responsible for generating ATP, which is an essential substance for all energy demanding processes within the cell which include; the performance of mechanical work, such as in muscle contraction and other cellular movements; the active transport of molecules and ions across cell membranes; and the chemical biosynthesis of macromolecules and other biomolecules from simple precursors. Therefore, any mutation which effects the transcription or translation of the genes which encode for the subunits of the respiratory enzyme complexes may result in a deleterious reduction in energy supply to the cell leading to a decline in physiological function. Whilst it is acknowledged that mutations of both the nuclear genome and the mitochondrial genome may effect the respiratory complexes of the oxidative phosphorylation system, it has been found that the mtDNA genome has a number of characteristics which render it more susceptible to mutation than the nuclear DNA.

(i) MtDNA is a naked molecule with a high informational density:

In contrast to the nuclear genome, mtDNA is not extensively covered with proteins, for example histones (Salazar et al 1982), which leaves the genome unprotected and open to insults from various genotoxic species. It is also generally recognized that mtDNA is tightly packed with functional reading frames, where each gene overlaps the next (Linnane et al 1989, Anderson et al 1981). As a result, any mutation which does occur within the mtDNA will involve a functionally important part of the genome. The nuclear genome differs in that only 10% of the entire genome has been assigned a functional role.

(ii) MtDNA is replicated with low fidelity:

The mammalian DNA polymerase (gamma) responsible for mtDNA replication, does not appear to replicate the DNA with high fidelity, which results in a higher mutation rate being observed in the mitochondrial genome compared to that of the nuclear DNA

(Brown et al 1979). Matsukage and coworkers (1975) reported that this was due to the fact that the polymerase lacks proof reading exonuclease activity. Kunkel and Loeb (1981) subsequently calculated the misinsertion rate of the mtDNA polymerase to be approximately 1/7000. Since the entire genome is 16.5Kb, this would imply 2-3 mismatched nucleotides (point mutations) to occur within each mtDNA molecule per round of replication. In addition, since the mtDNA genome is synthesized continuously throughout the cell cycle (Bogenhagen & Clayton 1977, Williamson & Moustacchi 1971) even when nuclear DNA replication is not occurring, mtDNA is likely to accumulate more mutations during each cell cycle.

(iii) MtDNA is continuously exposed to a flux of reactive oxygen species:

The transient attachment of the mtDNA genome to the inner mitochondrial membrane positions it in close contact with the mitochondrial electron transport chain which is the site where the majority of the reactive oxygen species (ROS) are formed within the cell (Chance et al 1979, Cadenas 1989). ROS are produced as byproducts during the four step univalent electron reduction of molecular oxygen into water, since the electron flow process of the mitochondrial electron transport chain is not fully efficient (Boveris et al 1972). By sequential one electron reductions of oxygen a series of reactive species are formed which include the superoxide radical ($O_2^{-}\cdot$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$), and singlet oxygen (O^{-}). Calculations show that during normal metabolism, one rat liver mitochondrion produces approximately 3 x 10^7 superoxide radicals per day (Richter et al 1988), and Forman and Boveris (1982) estimated that the steady state concentration of mitochondrial superoxide and hydrogen peroxide was in the picomolar and nanomolar range respectively.

Since oxygen radicals are short lived (Sawyer 1988) they attack molecules which are close to their site of production, hence the mtDNA would be continuously exposed to ROS, and due to being unprotected by proteins, free radical induced mutations would be likely to occur (reviewed by Richter 1992). The effect of these

ROS upon the mtDNA can occur by two pathways; 1) ROS can attack the DNA directly via reacting with either the sugar phosphate backbone which leads to strand fragmentation, or to the bases themselves resulting in the formation of damaged bases (eg base adducts) (Imlay & Linn 1988, Halliwell & Aruoma 1991), or 2) ROS can cause DNA damage indirectly by causing damage to other macromolecules such as the enzymes involved in DNA repair and replication, or they may initiate lipid peroxidation whose byproducts are known to be genotoxic (Pietronigro et al 1977, Akasaka 1986).

Numerous studies have shown that ROS are capable of directly attacking the mitochondrial genome. Richter et al (1988) were one of the first groups to measure the steady state level of oxidized bases in mtDNA in comparison to the nuclear genome, where they looked at the most commonly studied oxidized base 8-hydroxy-deoxyguanosine (8OHdG), which is formed by the hydroxylation of the deoxyguanosine residue at the C-8 position (Halliwell & Aruoma 1991). They calculated that the level of 8OHdG in nuclear DNA of rat liver was approximately 1/130 000 bases in contrast to the mtDNA which had a level of 1/8000 bases. Thus, this study indicated that the steady state level of oxidized bases in mtDNA is approximately 16x higher than the nuclear DNA. Upon treatment of various pro-oxidants, these steady state levels were found to increase which supports the assumption that the base adduct 8OHdG is a byproduct of oxy radical induced damage. In addition, besides being a useful marker of oxidative damage, the formation of 8OHdG has also been shown to be mutagenic, causing misreading at the 8OHdG residue itself and at neighboring bases resulting in G to T, and A to C substitutions (Kuchino et al 1987, Wood et al 1990, M^{c} Bride et al 1991, Cheng et al 1992, and Fraga et al 1990).

ROS can also generate strand breaks in the mtDNA. For example, bleomycin and adriamycin introduce nicks in mtDNA in an oxygen dependent manner in vivo, in cell cultures, or in isolated mitochondria, and extensive strand fragmentation of mtDNA has also been found in isolated mitochondria exposed to the redox cycler alloxan (reviewed by Richter 1988, 1992). It is therefore reasonable to assume that ROS may

be agents responsible for the observed base modifications and fragmentation of mtDNA in vivo.

As previously mentioned, ROS can also have an indirect action on the mitochondrial genome via initiating lipid peroxidation, which is a autocatalytic reaction that extends the destructive potential of free radicals. The inner lipid mitochondrial membranes are the closest molecules to the reactive oxygen species generated by the mitochondrial electron transport chain, thus it is not suprising that they are the prime target for most of the initial damage created.

During lipid peroxidation, a variety of reactive intermediates are formed which include carbon centred lipid radicals (L·), lipid peroxyl radicals (LOO·) and lipid alkoxyl radicals (LO·). Termination of this peroxidative decomposition of membrane lipids occurs when free radicals arising from this system are eliminated through radical-radical interaction, which may lead to intermolecular crosslinking. In vitro experiments show that mtDNA is damaged when mitochondria undergo lipid peroxidation, and there is evidence that when lipid radicals contact DNA they react with it (Pietronigro et al 1977, Akasaka 1986).

These initial studies lead to experiments where mtDNA was isolated from mitochondria which had been incubated under conditions in which lipid peroxidation was either stimulated or prevented (Hruszkewycz 1988, Hruszkewycz & Bergtold 1990, Balcavage 1982). These studies discovered a strong association between mitochondrial lipid peroxidation and the loss of the normal banding pattern of undigested mtDNA on agarose gels. Hruszkewycz (1989, 1990) found that addition of scavengers for superoxide anion, hydrogen peroxide and hydroxyl radicals (superoxide dismutase, catalase, and mannitol respectively), offered no protection against either lipid peroxidation or mtDNA damage. However, the addition of alpha tocopherol (an antioxidant that inactivates lipid radicals) gave simultaneous protection against both lipid peroxidation and DNA damage. These results support the view that the mitochondrial peroxidative decomposition of membrane lipids plays an important role in causing mitochondrial genetic damage. These experiments also support the

hypothesis that ROS may mediate mtDNA damage via indirect mechanisms using lipid peroxidation products as an intermediate link.

(iv) MtDNA is sensitive to various chemicals:

The histone free, supertwisted, circular structure of mtDNA favors the binding of various chemical carcinogens (eg alkylating agents) (Bandy & Davison 1990). The high lipid content of mitochondria also creates a high lipid:DNA ratio which renders the mtDNA particularly susceptible to lipophilic chemicals which can accumulate in lipid membranes where mtDNA and its replicating enzymes are localized.

Numerous studies involving the exposure of cells or animals to carcinogens have shown that chemical carcinogens result in modifications of the mitochondrial genome, and do so to a far greater extent than to the nuclear genome. For example, intraperitoneal injection of N-(^{14}C) methylnitrosourea (NMU) and N-nitroso (^{14}C) dimethylamine (DMNA) have been found to bind to mtDNA of rat liver cells approximately 5x greater than to nuclear DNA (Wunderlich et al 1970, 1971). Polycyclic aromatic hydrocarbons (PAHs) have also been found to bind to mtDNA, where Backer & Weinstein (1980) uniformly labelled mouse embryo cells and rat liver epithelial K16 cells with dihydrodiol epoxide derivative of benzo (alpha) pyrene, and found that the carcinogen labelling of mtDNA was 40-90 times more than that of the nuclear genome. In addition, Allen & Coombs (1980) examined PAH's of differing carcinogenicity and found them to bind to mtDNA of cultured mouse embryo cells 50-500 times more readily than to nuclear DNA. Administration of aflatoxin (Niranjan et al 1981), and carbontetrachloride (Levy & Brabec 1984) to experimental animals have also been found to preferentially covalently bind to liver mtDNA 3-4 and 20-50 times more than to the nuclear genome respectively.

In addition to binding to the mtDNA, various chemicals have been shown to induced single strand breaks within the genome (Wilkinson et al 1975, Miyaki et al 1977), and cause oxidative DNA base adduct formation (Hayakawa et al 1991a). For

Although there is evidence to support that some DNA repair mechanisms are operational within the mitochondria (Anderson & Friedberg 1980, Gupta & Sirover 1981, Domena & Mosbaugh 1985, Tomkinson et al 1988, 1990, Levin & Zimmerman 1976, Myers et al 1988, Satoh et al 1988), there are also other studies (Clayton et al 1974, Prakash 1975, Niranjan et al 1981, Lansman & Clayton 1975, Croizat & Attardi 1975, Miyaki et al 1977) which have shown that they are inadequate to repair all genetic damage which arises in this mitochondrial genome.

example, the addition of dimethyl nitrosamine resulted in a decrease in the amount of mtDNA which could be isolated in the closed circular form with a corresponding increase in the level of open circular or linear forms of mtDNA. Wilkinson et al (1975) attributed these observations to single strand breaks of the normally supercoiled DNA. In addition, DNA base modifications within mtDNA were observed after 4 weeks administration of azidothymidine (AZT) to mice (Hayakawa 1991a), which caused an increase in the number of hydroxylated deoxyguanosine residues in mouse liver mtDNA. These levels reached values as high as 38% of the total guanosine nucleotides after administration of doses equivalent to half of that given to AIDS patients.

(v) Insufficient DNA repair mechanisms within the mitochondria:

So far, the susceptibility of the mitochondrial genome to attack by genotoxic species has been addressed however, the question which remains is whether the efficiency and capacity of the repair mechanisms within the mitochondria are sufficient to cope with such insults to their genomes. Numerous studies have shown that these cytoplasmic organelles lack excision repair and recombinational DNA repair (Clayton et al 1974, Prakash 1975). Thus, it has been suggested that mitochondria are lacking adequate repair mechanisms which are known to be active within the nucleus (Friedberg 1985, Ames et al 1990).

There have been various reports concerning the lack of mitochondrial repair, and many have highlighted the fact that surviving cells have a higher level of unrepaired mutations in the mitochondrial genome than in contrast to the nuclear DNA. After interperitoneal administration of aflatoxin to rats (Niranjan et al 1981), the level of adducts within the mtDNA remained nearly constant for 24 hours, inhibiting mitochondrial transcription and translation, whereas the level of nuclear DNA adducts declined to zero.

Clayton et al (1974), also observed a lack of mtDNA repair when pyrimidine dimers were induced by UV irradiation into mtDNA of mouse L cells, human K13, and

HeLa cells. These dimers were not removed, indicating poor excision repair mechanisms. In addition, single strand breaks introduced into mtDNA of mouse cells by incubating them with bromodeoxyuridine and exposing them to visible light were not repaired even after 36 hours of post irradiation incubation (Lansman & Clayton 1975, Croizat & Attardi 1975). Similar results were also observed by Miyaki and coworkers (1977) who found a lack of repair 20 hours after the administration of 4-nitroquinoline oxide or N-methyl-N-nitro-N-nitrosguanidine to HeLa S3 cells.

Therefore, these observations reflect minimal repair of the mtDNA damage, in contrast to the more efficient DNA repair mechanisms which exist within the nucleus. Recent studies have suggested that some DNA repair mechanisms may be operational within the mitochondria, which involve 3 uracil DNA glycosylases (Anderson & Friedberg 1980, Gupta & Sirover 1981, Domena & Mosbaugh 1985), 2 endonucleases specific for apurinc/apyrimidinic sites (endonucleases which act at lesions introduced by high UV doses) (Tomkinson et al 1988, 1990), and a DNA ligase (Levin & Zimmerman 1976). Also, Myers et al (1988) and Satoh et al (1988) have reported the repair of some alkylated bases. However, this evidence that some repair mechanisms exists within the mitochondria appears to be limited and requires further investigation.

1.1.4. Mutations of the mitochondrial genome:-

Due to the susceptibility of the mitochondrial genome to mutations and the inadequate repair mechanisms, it would seem likely that mutations of this genome are a common occurrence. Due to the high copy number of mtDNA molecules within the cell, when a mtDNA molecule acquires a mutation, this would create a mixed intracellular mitochondrial gene pool of both mutant and wildtype molecules which is known as heteroplasmy. Mitochondria containing such mutated genomes could still continue to replicate because the genes which are important for mitochondria production are located within the nucleus, and the mitochondrial lipids, structural proteins and

other enzymes needed for replication of the mitochondria are produced by the cytoplasmic protein synthesizing apparatus. In addition, the mutated mitochondrial genome may also continue to replicate because the enzymes required for mtDNA replication are also encoded by the nuclear DNA and are subsequently imported from the cytoplasm into the mitochondria (Attardi 1981a, 1981b). Therefore, mitochondria containing mutated DNA may accumulate in cells where the mutation is amplified with each replication of the mitochondrial genome.

Upon cell division it has been widely proposed that mitochondria and consequently mtDNA are randomly partitioned into daughter cells, resulting in some lineages drifting toward pure mutant mtDNAs, others toward pure wildtype mtDNAs, while others still remain heteroplasmic. However, a recent study by Hayashi et al (1994) has shown that mitochondria within the same cell interact with each other, and it appears that mitochondrial DNA molecules may be transferred between mitochondria within the same cell. Therefore, this rapid diffusion of mtDNA and the resultant homogeneous distribution of the heteroplasmic wildtype and deleted mtDNA molecules throughout mitochondria in cells suggests that mitochondria in cells have lost their individuality, and can be considered as a single functional unit. Therefore, the presence of a mutated genome in any particular mitochondrion may be shared between all the mitochondria within the same cell. Thus, upon cell division, a copy of the mutated genome could separate into both daughter cells.

In cells harboring mutant mtDNAs, the cell phenotype would be a product of the nature of the mtDNA mutation, the proportion of mutant mtDNA within the cell (degree of heteroplasmy), and the degree to which the cell type relies on mitochondrial OXPHOS function. It is proposed that clinical symptoms would develop when the mitochondrial energy output falls below the minimum threshold level necessary for a tissue to function normally. These symptoms would vary widely since different tissues rely on mitochondrial energy to different extents, where the organ system most dependent on mitochondrial energy is the central nervous system (CNS), followed by skeletal muscle, cardiac muscle, kidney and liver.

It is plausible that the accumulation of damages to the mitochondrial genome during a human's lifespan would in turn cause mutation of certain genes or loss of mitochondrial gene products, which would be manifested as a decline in mitochondrial respiratory function. The decreases in the bioenergetic capability and capacity of the cell would lead to a concomitant decrease in ATP synthesis, falling below the energy threshold required for normal tissue function. Thus, it has been proposed that mutations of the mitochondrial genome contribute not only to the pathogenesis of mtDNA diseases but also to the deterioration seen with age in multiple tissues (Miquel 1992, Bittles 1992).

1.2.0. MtDNA mutations in association with mitochondrial diseases:-

A number of human diseases have been found to be associated with defects in oxidative phosphorylation resulting from alterations of the mitochondrial genome (Kadenbach 1990, Wallace et al 1991, 1992a, 1992b, 1993). These pathological mtDNA mutations fall into two major classes. Firstly, there are those diseases which have been found to be associated with point mutations within the mitochondrial genome, which include missense (amino acid) and protein synthesis (tRNA) mutations, which have been identified as maternally inherited and secondly, deletions and insertions within the mitochondrial genome have been identified within other disease states which appear to be mainly sporadic and not maternally inherited.

1.2.1. Disease related mtDNA nucleotide substitutions:-

(i) Missense mutations which alter electron transport chain polypeptides:

Point mutations within the mitochondrial DNA genome which result in alterations of the electron transport proteins have been identified. These maternally inherited mutations (Erickson 1972), primarily effect the CNS and result in the phenotypic expression of the disease state known as Leber's Hereditary Optic Neuropathy (LHON) which results in sudden adult onset blindness associated with degeneration of the optic nerve (Wallace 1992a, Wallace et al 1988a). These patients are generally normal throughout childhood, however with age they experience progressive bilateral vision loss which is usually the only clinical symptom, although cardiac conduction defects and minor neurologic abnormalities have been reported in some individuals (Newman & Wallace 1990, Newman 1991).

Multiple point mutations of the mtDNA have been described within this disease state (see Table 1.1), where different LHON families have been found to either harbor exclusively one type of mutant, or they may contain various combinations of these mutations which co-exist with the wildtype mtDNA population (review Brown et al 1992a). The most common LHON mutation which is present in 70% of patients is located at the basepair 11778 in the ND4 gene ($ND4^{11778}$), where a G to A transition is observed resulting in a highly conserved arginine amino acid being changed to a histidine (Wallace et al 1988a). This mutation was identified by restriction fragment length polymorphism studies (Wallace et al 1988a, Singh et al 1989), where it was usually found to be homoplasmic in the majority of LHON patients. The establishment of this ND4 gene mutation as the cause in LHON by Singh et al (1989), was the first direct demonstration that a mitochondrial DNA point mutation can result in human disease.

The second most frequently observed mutation, which is found in approximately 30% of LHON families who suffer from the disease, involves a G to A transition in the ND1 gene at the nucleotide position 3460 ($ND1^{3460}$) (Houponen et al

Table 1.1. Mitochondrial DNA base substitutions associated with mitochondrial diseases:- This table summarizes the gene and nucleotide positions of mitochondrial DNA base substitutions which have been found to be associated with various mitochondrial diseases, which include; Leber's Hereditary Optic Neuropathy (LHON), Neuropathy, ataxia, retinitus pigmentosa (NARP), Leigh's syndrome, mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS), myoclonic epilepsy and ragged red muscle fibres (MERRF), and maternal myopathy and cardiomyopathy (MMC). The gene position abbreviations are as follows; ND1, ND2, ND4, ND5 and ND6 correspond to subunits 1, 2, 4, 5 and 6 of the respiratory enzyme complex I (NADH dehydrogenase), COXI represents subunit I of cytochrome c oxidase (complex IV), Cyt. b corresponds to cytochrome b, a subunit of the respiratory complex III, ATPase 6 encodes for a subunit of complex V (ATPase synthase), and the remaining genes correspond to various transfer RNA (tRNA) genes. The nucleotide position is numbered according to the human mtDNA sequence derived by Anderson et al (1981).

Disease:	Gene mutated:	Position:	Base substitution:
LHON	ND1	3394	T→C
	"	3460	G→A
	"	4160	T→C
	"	4216	T→C
	ND2	4917	A→G
	"	5244	G→A
	COXI	7444	G→A
	ND4	11778	G→A
	ND5	13708	G→A
	ND6	14484	T→C
	Cyt.b	15257	G→A
	"	15812	G→A
NARP / Leigh's	ATPase 6	8993	T→G
MELAS	$tRNA^{Leu(UUR)}$	3243	A→G
	"	3271	T→C
MELAS / MERRF	$tRNA^{Lys}$	8356	T→C
MERRF	$tRNA^{Lys}$	8344	A→G
MMC	$tRNA^{Leu(UUR)}$	3260	A→G

1991, Howell et al 1991a,b). Within this same gene, a T to C substitution has also been detected within one large LHON family at the nucleotide position 4160 (NDI^{4160}). In addition, 17% of LHON families have been found to have a point mutation in the cytochrome b gene of complex III at the nucleotide position 15257 ($cyt.b^{15257}$), where a G to A substitution has been reported (Brown et al 1992a,b, Johns & Neufeld 1991).

These critical mutations account for the largest subset of LHON patients and individuals harboring these mutations have a relatively high probability of going blind, since they usually cause significant changes in evolutionary conserved amino acids within functionally important polypeptide domains. In addition, these mutations are found rarely, if at all, in individuals in the unaffected population. It has been proposed that these mutations are responsible for the phenotypic loss of vision by significantly reducing the efficiency of the electron transport chain. This is supported by the fact that the specific activity of complex I of the respiratory chain has been found to be reduced in patients harboring either the $ND1^{3460}$ or the $ND1^{4160}$ mutations (Howell et al 1991a, Howell et al 1991b, Majander et al 1991). A complex I enzymological defect has not been found in patients with the $ND4^{11778}$ mutation however, respiration studies have revealed a decreased oxidation of NADH-dependent substrates (Majander et al 1991, Larsson et al 1991). So far, no biochemical evidence exists for the complex III, $cyt.b^{15257}$ mutation.

Other point mutations which are present in LHON patients but are considered low risk LHON mutations include; a T to C transition in the ND1 gene at the nucleotide position 4216 ($ND1^{4216}$), an A to G substitution in the ND2 gene at the nucleotide position 4917 ($ND2^{4917}$), and G to A transitions at each of the nucleotide positions 5244, 13708 and 15812 which are found within the ND2, ND5 and the Cyt.b genes respectively ($ND2^{5244}$, $ND5^{13708}$ and $Cyt.b^{15812}$ respectively) (Brown et al 1992b, Wallace 1993). These mutations create both conserved and non-conserved amino acid substitutions and can be found rarely ($ND2^{5244}$), or frequently ($ND1^{4216}$) in the unaffected control population. The probability that an individual will go blind when harboring only these mutations is low however, these mutations are often found in

association with those more severe LHON point mutations, and it is proposed these less critical mutations may enhance the severity or increase the probability of expressing LHON (Johns & Berman 1991). For example, the $ND2^{5244}$ and the $Cyt.b^{15812}$ mutations are frequently found with the $cyt.b^{15257}$ mutation, and the incidence of blindness is increased with the addition of these less critical mutations (Brown et al 1992a).

There are also two other point mutations which cause significant alterations in the electron transport chain polypeptides, but they are relatively uncommon in LHON patients where they exist in isolated cases in association with the other point mutations already discussed. One of these involves a G to A transition within the COI gene at the nucleotide position 7444 (COI^{7444}) (Brown et al 1992c). This mutation converts the termination codon of the COI gene to a Lys codon, resulting in an extension of the CO1 polypeptide by 3 amino acids, which alters the mobility of CO1 on SDS-polyacrylamide gels and reduces the specific activity of cytochrome oxidase by 36% (Brown et al 1992c). The $CO1^{7444}$ gene mutation has been found in association with the severe $ND1^{3460}$ mutation, where it probably interacts to increase the likelihood of LHON expression. The second mutation is characterized by a T to C substitution at the nucleotide position 3394 which is located within the ND1 gene ($ND1^{3394}$) (Brown et al 1992a). This point mutation results in a highly conserved tyrosine amino acid being converted into a histidine, and it has been found in association with 7% of patients harboring the $ND4^{11778}$ mutation (Brown et al 1992a).

While the above evidence supports the involvement of mtDNA in LHON, there are a number of factors which suggests that mtDNA is not the sole factor involved in the pathogenesis. The most important being the fact that 85% of sufferers are male which indicates some involvement of the X-chromosome in the expression of the disease as proposed by Vilkki et al (1991). Also, the reason for the presence of some family members which harbor these mutations but remain visually asymptomatic while others develop the disease is unclear and this suggests that additional genetic (nuclear of

mitochondrial), environmental, or physiological factors play a significant role in the expression of this LHON disease state.

(ii) Missense mutations which effect ATPase Synthase:

The second class of maternally inherited base substitutions effect complex V of the oxidative phosphorylation system, by causing a T to G transition in the ATPase 6 gene (Holt et al 1990). This mutation results in a conserved hydrophobic leucine amino acid of ATPase 6 being substituted for a hydrophilic amino acid. This leucine normally occurs in a consistently hydrophobic region of the polypeptide, and therefore the substitution might be expected to affect the structure and function of the enzyme (ATPase synthase) in these patients. In contrast to the LHON mutations, this mutation is invariably heteroplasmic ranging from 9-97% of the total mtDNA population, hence this mutation can be associated with a striking variety of phenotypes depending on the proportion of mutant mtDNA's inherited by different maternal relatives (Holt et al 1990, Tatuch et al 1992, Shoffner et al 1992). For example, patients with relatively low levels of this mutation contributing to 10 to 50% of the total mtDNA population usually experience no symptoms, whilst a child which is born with 60-80% mutant mtDNA, will eventually develop the clinical phenotype known as NARP (neuropathy, ataxia, and retinitus pigmentosa) which is a debilitating but not fatal disorder (Holt et al 1990). However, if a child is born with extremely high levels of this ATPase 6 point mutation (>90%), it will experience a totally different pathological condition known as maternally inherited Leigh's syndrome, which is a devastating and fatal mitochondrial encèphalomyopathy (Tatuch et al 1992).

In patients with Leigh's syndrome, muscle biopsies have shown no histochemical evidence of mitochondrial myopathy however, a few patients show cytochrome oxidase deficiency and mitochondrial abnormalities on enzyme assays have been identified in approximately 25% of cases. In addition, deletions within the

mitochondrial genome have also been described in some Leigh's syndrome patients (Somer et al 1991).

(iii) Base substitutions which effect protein synthesis:

The final class of maternally inherited mtDNA point mutations involve a number of nucleotide substitution mutations which effect mtDNA protein synthesis (Wallace 1992a,b, 1993). These mutations have been found in the genes which encode for tRNA's, and are generally heteroplasmic causing multiple organ system failure. The best characterized of these tRNA mutations involve; 1) an A to G transition (at nt 8344) in the $tRNA^{Lys}$ gene (Shoffner et al 1990), which is associated with myoclonic epilepsy and ragged red fibre disease (MERRF), 2) an A to G transition (at nt 3243) in the $tRNA^{Leu(UUR)}$ gene (Goto et al 1990, Kobayashi et al 1990), which is associated with the disease state known as mitochondrial encephalomyopathy and stroke like episodes (MELAS), and 3) an A to G transition in the $tRNA^{Leu(UUR)}$ gene (Zeviani et al 1991), which is associated with maternally inherited myopathy and hypertrophic cardiomyopathy (MMC).

Patients with MERRF have abnormalities within the CNS and skeletal muscle which are characterized by limb weakness, ataxia and myoclonic seizures. The finding that several MERRF pedigrees exhibit clear maternal inheritance (Rosing et al 1985), and the observation that most patients have reduced activity of the respiratory complexes I and IV in skeletal muscle mitochondria, suggested that an abnormality of the mitochondrial genome may be contributing to this phenotype. Subsequent experiments by Shoffner et al (1990), confirmed that an A to G point mutation at the nucleotide position 8344 in the $tRNA^{Lys}$ gene was associated with this disease state, and Seibel et al (1991) and Wallace et al (1988b) found that the presence of this mutation resulted in impairment of mitochondrial DNA protein synthesis. Although this mutation was found to be specific for MERRF pedigrees, it was not present in all MERRF patients and recently Wallace and coworkers (1993) reported that

a relatively less common point mutation, involving a T to C transition (at 8356) is also associated with this disease state.

The variation in severity of the biochemical and physiological deficiencies observed along the maternal lineage indicated that these mutations were heteroplasmic and that various maternal relatives inherited different proportions of mutant and wildtype mtDNA's. The order to which various tissues were affected was consistent with their relative reliance on mitochondrial energy production (Wallace et al 1988a), and a recent study by Hammans et al (1991) was successful in identifying the 8344 mutation in leukocyte and platelet mitochondria, which highlights the potential use of blood in the diagnosis of this disease.

MELAS patients usually have onset in childhood of growth retardation, seizures, ataxia, dementia, deafness, mild limb weakness, and stroke-like episodes with resting lactic acidosis. Matthews et al (1994) found that this mutation within a fetus displayed very little variation in heteroplasmy in different tissues, in contrast to adult tissues where the proportion of mutated genomes varied between different tissues from the same subject and between the same tissue in different subjects. Matthews et al (1994) suggested that selection pressures acting on either the wildtype or the mutant mtDNA may be responsible for the variable levels of 3243 mutant mtDNA being observed in different heteroplasmic tissues of adults. Also, Yoneda et al (1992) reported that mtDNA molecules carrying this MELAS mutation have a replicative advantage over the wildtype molecules which may account for the observed increase in level of mutant genomes seen in the adult tissues.

Complex I deficiency is the most common respiratory chain defect found in MELAS patients, although multiple deficiencies involving complexes I-IV have also been described (Goto et al 1992). In addition, the MELAS $tRNA^{Leu\ 3243}$ mutant has been shown to cause serious defects in mitochondrial protein synthesis and respiration in cell lines carrying it in the homoplasmic form (King et al 1992, Chomyn et al 1992). Hess and colleagues (1991) recently proposed that whilst this mutation could effect the function of the tRNA, it may also affect rRNA transcription since this mutation occurs

at the mtDNA binding site of a nuclear encoded protein which terminates rRNA transcription.

In addition to this $tRNA^{Leu3243}$ mutation, two less common point mutations have been also been associated with this disease state. One of these involves a T to C mutation at the nucleotide position 3271 within the same $tRNA^{Leu}$ gene (Hayashi et al 1993), whilst the other is present within the $tRNA^{Lys}$ gene at the nucleotide position 8356, which is the same mutation which has also been identified within a few MERRF patients (Wallace 1993).

1.2.2. Disease related mtDNA deletions:-

In contrast to the previously described disease-related point mutations, the majority of patients which carry deleted mtDNA molecules have no family history suggestive of mitochondrial disease. Neither the mothers nor the siblings of these patients are clinically affected, nor do they harbor deleted mtDNA's. The presence of these disease related deleted molecules usually occurs spontaneously, where the deleted segment usually removes any part of the genome except for the regions spanned by either of the replication origins. High levels of these subgenomic mtDNA molecules have been observed in patients with Pearson's marrow / pancreas syndrome (Rotig et al 1988, 1989, 1990) and various ocular myopathies which include Kearn's Sayre syndrome (KSS) and chronic progressive external opthalmoplegia (CPEO) (Wallace 1992a,b, 1993, Schon et al 1989). Whilst the same deletions are present in the genomes of patients with both of these diseases, the main difference is that KSS and CPEO patients usually harbor a large proportion (up to 80% of the total mtDNA) of deleted mtDNA's in muscle, but very few (<5%) in blood, whereas in Pearson's syndrome, the figures are almost exactly reversed.

Pearson's syndrome is generally a fatal childhood disorder involving pancytopenia caused by reduced numbers of bone marrow precursor cells, pancreatic

fibrosis, and splenic atrophy. Individuals who survive their pancytopenia usually develop the symptoms of KSS in early adolescence, as the proportion of deleted mtDNA's increases in their muscle mitochondria with age (Larsson et al 1990), whilst at the same time the levels decline in their blood cells following a series of therapeutic blood transfusions (Rotig et al 1988, 1989, Norby et al 1994). Patients with KSS or CPEO are clinically variable, though all are characterized by opthalmoplegia (paralyses of the eye muscles), ptosis (droopy eye lids), mitochondrial myopathy and usually have onset before the age of 20. Mild cases of these ocular myopathies which only have these symptoms are designated as CPEO, whereas severe cases which may also have other symptoms such as retinitis pigmentosa, hearing loss, and heart conduction defects are designated as KSS (Holt et al 1988).

Holt et al (1988), Lestienne et al (1988) and Zeviani et al (1988) were among the first to report that deletions ranging from 1.3Kb to 7.6Kb were present within the muscle mtDNA genomes of patients with mitochondrial myopathies. These deleted molecules were found to co-exist with the wildtype mtDNA population, where the size and position of the deletion varied between different patients. Moraes et al (1989) subsequently noted that despite this size variability, all the deleted segments preserved the regions spanned by the promotors of transcription, the 12S and the 16S rRNAs, and the origin of replication of the heavy strand, where the majority of the deletions were found to lie within the major region between the two origins of replication. In contrast, all the tissues in any one individual were found to harbor a single specific type of deletion, which suggests that the deleted mtDNA population in any one patient is a clonal expansion of a single spontaneous deletion event which occurred early in oogenesis or embryogenesis.

The most frequently observed deletion, which is termed "the most common deletion", is a 4977bp deletion which extends from the ATPase 8 gene to the ND5 gene (see Diagram 1.4). This 5Kb deletion results in the removal of several genes which include; ND3, ND4, ND4L, ND5, COX III, ATPase 6 and 8, and several tRNA genes. In a study of 30 patients with KSS or CPEO, 12 patients were found to have an

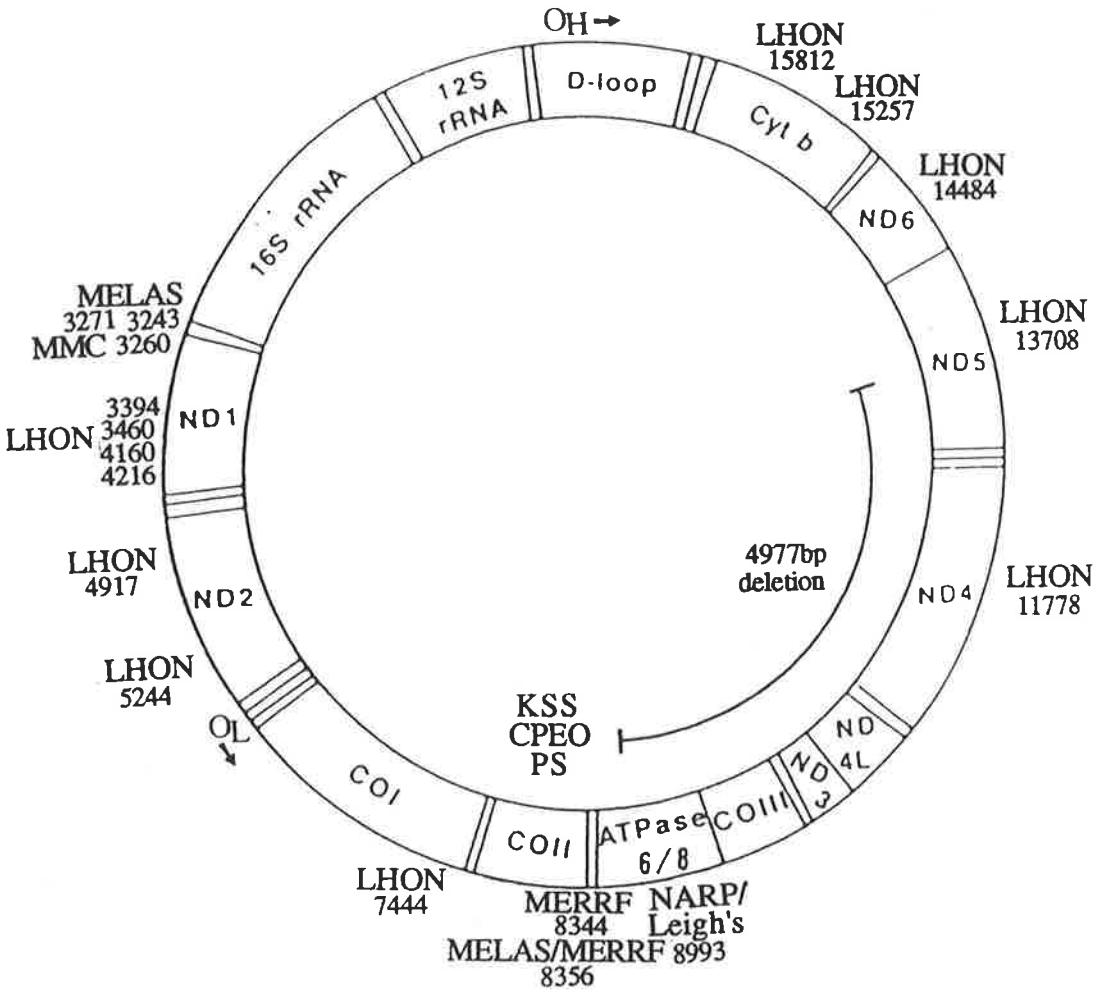


Diagram 1.4. Mitochondrial DNA mutations associated with mitochondrial diseases:-
This diagram summarizes the gene location of mtDNA base substitutions and the "most common" deletion which are associated with various mitochondrial diseases. The abbreviations for the disease states are as follows; Leber's Hereditary Optic Neuropathy (LHON), Neuropathy, ataxia, retinitus pigmentosa (NARP), Leigh's syndrome, mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS), myoclonic epilepsy and ragged red muscle fibres (MERRF), Maternal myopathy and cardiomyopathy (MMC), Kearn's Sayre syndrome (KSS), Chronic progressive external opthalmoplegia (CPEO) and Pearson's marrow / pancreas syndrome (PS). The nucleotide positions are numbered according to the human mitochondrial genome sequence derived by Anderson et al (1981). The open bars represent transfer RNA (tRNA) genes; 12SrRNA and 16SrRNA are ribosomal RNA genes; ND1, ND2, ND3, ND4L, ND4, ND5 and ND6 genes, encode for subunits 1, 2, 3, 4L, 4, 5 and 6 of NADH dehydrogenase (complex I); COI, COII and COIII genes encode for subunits 1, 2 and 3 of cytochrome c oxidase (complex IV); cyt b gene, encodes for cytochrome b which is a subunit of Ubiquinol-cytochrome c oxidoreductase (complex III), and ATPase 6/8 genes encode for subunits 6 and 8 of ATPase synthase (complex V). The light and heavy strand origins of replication are denoted as O_L and O_H respectively.

identical deletion which corresponds to this most common 4977bp deletion (Holt et al 1989a). Independently, Rotig et al (1989, 1990) confirmed the presence of this same deletion in blood (lymphocytes) samples obtained from patients suffering from Pearson's marrow syndrome, and suggested that this site was a hot spot for deletion.

An important feature of these disease related mitochondrial DNA deletions is that they all result in numerous tRNA genes being removed from the genome. This should result in a partial deficiency in mtDNA translation, curtailing the expression of all mtDNA proteins and hence affect all four respiratory complexes I, III, IV and V. Therefore, since any sizeable deletion is bound to include a tRNA gene and consequently prevent translation of the remaining mitochondrial genes, the entire mitochondrial genome may function as a single genetic unit rather than a series of genes encoding for individual enzymes. Thus, this concept would explain why patients with different deletions can experience the same phenotype. Nakase and colleagues (1990), whilst studying patients with large deletions, showed that deleted mtDNA's are transcribed but protein synthesis does not take place. This is not suprising since the molecules involved in the transcription of mtDNA are nuclear encoded, while the tRNA's and rRNA's required for translation are encoded on the mitochondrial genome.

Holt et al (1989a,b) subsequently calculated that the proportion of affected mtDNA molecules in skeletal muscle of patients with mitochondrial myopathies, ranged from 20 to 90% of the total mtDNA population. To account for such high levels of deleted mtDNA molecules in the cell it has been proposed that they have a replicative advantage. This theory is supported by the fact that all the proteins necessary for mtDNA replication are encoded by nuclear genes and therefore the rate of replication of individual molecules within a cell would be directly proportional to their length. Therefore, the deleted, smaller molecules would replicate more rapidly than the normal wildtype genomes, and increase with each cell generation. This intracellular replication competition would explain the predominance of deleted molecules within these patients, and could explain the delayed onset of neurological diseases both in patients with Pearson's syndrome and in others with mitochondrial myopathy.

In contrast to blood samples obtained from Pearson's syndrome patients (Rotig et al 1988), Southern blotting and restriction enzyme digestion analyses have failed to demonstrate the presence of mtDNA with deletions in fast replicating cells such as those found in blood from patients with mitochondrial myopathies. However, using PCR analysis, where the primers were positioned outside of the deletion breakpoints, Poulton et al (1991) was successful in detecting a minor proportion of partially deleted mtDNA in blood cells obtained from patients with mitochondrial myopathies. Recent advances in PCR technology have also made it possible to identify subgenomic (deleted) mtDNA molecules bearing deletions in patients with cardiomyopathy (Ozawa et al 1990b, Takeda et al 1993). The most common deletion detected was a 7436bp deletion and Ozawa and coworkers (1990b) estimated that the level of mutated genomes was less than 10% of the total mtDNA population. In addition, Suomalainen et al (1992a) observed multiple deletions within the mitochondrial genomes of patients with inherited idiopathic dilated cardiomyopathy (DCM), which is characterized by dilatation and dysfunction of one or both ventricles.

In some KSS pedigrees, the disease has been found to be inherited as an autosomal-dominant mutation, in which affected members harbor multiple mtDNA deletions, instead of a single deletion which is found in spontaneous cases of the disease (Zeviani et al 1989, 1990). In these pedigrees, the trait was autosomal dominant because transmission was through both paternal and maternal lineages and both sexes were affected in subsequent generations. This would suggest that these patients have a mutation within a nuclear gene which is essential for replication or maintenance of mtDNA.

In addition to deletions, three partial duplications within the mitochondrial genome of 8Kb have been reported in patients with KSS (Poulton et al 1989, Brockington et al 1993) and Pearson's syndrome (Rotig et al 1990). These duplications involve the heavy strand origin of replication, and the range of heteroplasmy has been found to differ in different tissues. Poulton et al (1989) also found that these duplications could be maternally inherited. The mechanism by which such duplicated

molecules are generated is unclear however, Grossman (1990) suggested that they could be the result of a partial deletion within a circular dimer, which is where two mtDNA molecules are joined in a head to tail arrangement.

1.2.3. Direct repeat sequences associated with mtDNA deletions:-

Mapping the position of the disease related deletions within the mitochondrial genome has revealed that approximately 50% of these are flanked by tandem direct repeat sequences which range in length from 5 to 13bp (Harding & Hammans 1992, Johns et al 1989). The most common deletion of 4977bp has been found to be associated with a 13bp direct repeat (Schon et al 1989). Relative to the direction of heavy strand replication, the first repeat is retained within the genome while the second repeat is deleted. Thus, the involvement of these direct repeat sequences has been implicated in the mechanism of formation of these deletions. Shoffner et al (1989) and Madsen et al (1993) suggested that a slip replication mechanism may take place, since the majority of the direct repeats which are associated with deletions are confined to the major region between the replication origins, and this region is exposed as a single strand for considerable periods during replication (Nelson et al 1989), so that slip-mismatches may well occur in this area. Due to the vast number of relatively short direct repeat sequences within the mitochondrial genome, these sequences could provide numerous chances for mtDNA mutations.

Mita et al (1990) subsequently proposed that the formation of deletions may involve recombination events mediated by enzymes recognizing short homologies. However, this proposal is not favored since mtDNA does not appear to have orthodox DNA repair systems. Also, the sequencing of other deletion borders has revealed partial direct repeats and in some cases there were no detectable repeats. Therefore, Degoul et al (1991) proposed that more than one mechanism may be involved in the production of

these deletions. For example, it is possible that mtDNA deletions may be due to a defect in a nuclear gene which results in altered replication of the mitochondrial genome.

1.2.4. Deleted mtDNA molecules and cytochrome c oxidase deficiency:-

Respiratory chain deficiencies have been found in complexes I, IV and V of patients with KSS (Bresolin et al 1987, Petty et al 1986, Shoffner et al 1989, Holt et al 1989b). In particular, histochemical studies have shown that deletions of the mitochondrial genome are related to focal cytochrome c oxidase (COX) deficiency in the skeletal muscles of patients with mitochondrial myopathies (Collins et al 1991, Mita et al 1989, Moraes et al 1992, Oldfors et al 1992, Shoubridge et al 1990, Prelle et al 1994). These authors mapped the distribution and expression of wildtype and deleted mtDNA molecules in skeletal muscle fibres from these patients and found an over-expression of deleted mtDNA molecules in muscle fibre segments which displayed negative COX (complex IV) activity. In contrast, the results obtained concerning the distribution of the wildtype mtDNA molecules varied. Shoubridge et al (1990) and Collins et al (1991) independently found that the level of wildtype molecules in these COX deficient fibres were near normal and suggested a functional dominance of deleted mtDNA over normal mtDNA. In contrast, Mita et al (1989), Prelle et al (1994) and Oldfors et al (1992) reported a low level of normal transcripts and suggested that this lack of wildtype mtDNA transcripts was responsible for the observed COX deficiency seen in these fibres.

1.2.5. MtDNA mutations associated with diabetes mellitus:-

Recent evidence has suggested that some cases of diabetes mellitus may result from multiple mtDNA mutations which involve both point mutations and deletions

(Kadowaki et al 1993, 1994, Ballinger et al 1992). Analysis of a large pedigree with maternally inherited diabetes and deafness revealed a generalized muscle mitochondrial respiration deficiency associated with a severe mitochondrial protein synthesis defect. Molecular analysis revealed that affected individuals had a systemic, heteroplasmic, 10.4Kb mtDNA deletion which was flanked by a 10bp direct repeat sequence located within the ND2 gene and the cytochrome b gene (Ballinger et al 1992). Thus, this deletion removed the origin of replication of the light strand of the genome and all of the mitochondrial genes except for the rRNA's, ND1, part of cytochrome b and the adjacent tRNA's. The resulting deficiency in respiration due to this large deletion, appeared to inhibit insulin production by the pancreatic islets.

In addition, a point mutation within the mtDNA genomes of patients with diabetes mellitus has also been observed in peripheral blood leukocytes (van den Ouweland et al 1992, Reardon et al 1992, Kadowaki et al 1993, 1994). This mutation involves an A to G transition within the $tRNA^{Leu}$ gene at the nucleotide position 3243, which has been associated with patients suffering with MELAS as previously discussed. This $tRNA^{Leu(UUR)}$ mutant DNA was found to exist in heteroplasmy with the normal wildtype genome, and Kadowaki et al concluded that this mutation played an important role in the manifestation of this disease since it is responsible for altering a highly conserved amino acid residue and was not detected in a large group of controls.

1.2.6. MtDNA mutations associated with degenerative diseases:-

Alterations in mitochondrial genetics are now being implicated in numerous degenerative disease states which include; Parkinson's disease (PD), Alzheimer's disease (AD) and Huntington's disease (HD) (reviewed by Schapira 1992). Deficiencies of the respiratory complex I have been reported in PD brains and platelet mitochondria (Schapira et al 1989, 1990, Mann et al 1992) however, studies on skeletal muscle from PD patients has generated conflicting results where some studies have

shown multiple respiratory chain deficiencies, some pure complex I deficiencies, and other report normal enzyme activities (Bindoff et al 1989, Shoffner et al 1991, Hattori 1991a). Ikebe et al (1990) and Ozawa et al (1990a) reported that deleted mtDNA molecules may be associated with this disease however, subsequent studies by Mann et al (1992) have shown that these results were due to an age related phenomenon which will be discussed later. Also pedigree analysis has failed to detect strong evidence that this disease is maternally inherited (Maraganore et al 1991), which disfavors the involvement of mtDNA in the manifestation of this disease state.

Alzheimer's disease (AD) is a common late onset disease associated with progressive dementia and cortical atrophy. Patients with this disease have been reported to have OXPHOS complex IV defects in platelet mitochondria (Parker et al 1990a), which suggests that this disease could be the product of mutations within the mitochondrial genome. However at this stage, there is no definite evidence of any mtDNA mutations being associated with this disease state. Blanchard et al (1993) recently investigated the presence of the 4977bp deletion within the brains of patients with AD and found that while these brains contained deleted molecules, the levels found were not significantly different from their age matched controls. Hence, the involvement of mtDNA defects with this disease state requires further investigation.

Huntingdon's Disease (HD) is a rare movement disorder associated with degeneration of the basal ganglia. The association between mtDNA mutations and this disease has also generated some interest. Due to a greater number of off spring of effected mothers developing this disease than those of effected fathers, it was proposed that this disease was linked to the mitochondrial genome. Defects within OXPHOS complexes I and IV have been revealed in basal ganglia, and in platelet mitochondria by enzyme assays (Parker et al 1990b), in contrast to other studies which have reported normal complex I activity within the caudate nucleus (Mann et al 1990), and normal restriction length polymorphism patterns (Irwin et al 1989). Thus, further investigation is required to confirm whether the mitochondrial genome is associated with this disease state.

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

Linnane and coworkers (1989) were among the first to suggest that mitochondrial DNA mutations and consequential changes in cellular energetics, may offer a significant contribution to the ageing process. This hypothesis was based upon the accumulating evidence published by other researchers who demonstrated that mtDNA molecules do get mutated or damaged otherwise during cell ageing in various organisms (Harman 1983, Miquel et al 1983, Fleming et al 1985, Piko et al 1988, Richter et al 1988, Bandy & Davison 1990). Linnane's theory of ageing suggested that the ageing process is caused by a time dependent accumulation of somatic mutational events affecting the intracellular gene pool of mtDNA molecules, and proposed that during cell division, random segregation of mtDNA molecules from this pool would result in tissue bioenergy mosaics. They also proposed that with time there would be a progressive depletion of energy production by the mitochondrial oxidative phosphorylation system resulting in a gradual decline of the physiological and biochemical performance of organs, significantly contributing to the ageing process and ultimately resulting in death.

At about the same time, Trounce et al (1989), Cardellach et al (1989) and Yen et al (1989) independently demonstrated that mitochondrial respiratory functions decline with age in human skeletal muscle and liver tissues respectively. Trounce et al (1989) calculated a significant negative correlation between respiration rate in skeletal muscle and age, and found that the activity of the respiratory enzyme cytochrome c oxidase (complex IV) also declined in muscle homogenates obtained from elderly individuals. Subsequent analysis of human skeletal muscle specimens confirmed this decline in activity of complex IV and in addition revealed a decrease in activity of complex I with respect to age (Cooper et al 1992, Boffolli et al 1994). Interestingly, whilst both of these groups observed that the activity of complex III remained relatively constant, Boffoli et al (1994) discovered that the level of cytochrome b, a mitochondrial encoded subunit of this complex, declined with advancing age. In addition, Boffoli et al also

reported a decline in activity of complex II, which indicates the involvement of the nuclear genome in the age-related decline in respiratory activity.

Examination of respiratory enzyme activities in other species has also revealed the same general pattern. Complexes I and IV have been shown to decline in the brains of Rhesus monkeys (Bowling et al 1993) and rats (Harmon et al 1987, Curti et al 1990, Torii et al 1992a), and in the skeletal muscle and hearts of rats and dogs (Sugiyama et al 1993). Sugiyama et al (1993) observed that a decline in activity of complexes I and IV in the rat skeletal muscle was significant after 28 weeks and 55 weeks respectively, whilst a significant reduction in these activities in the heart was only detected in rats of 100 weeks old, indicating that skeletal muscle mitochondria are more susceptible to ageing. None of these studies reported a significant decline in the activities of the respiratory complexes II, III and V.

The finding that the activities of complexes I, IV and cytochrome b decrease with age is consistent with the involvement of mtDNA defects since the mitochondrial genome is responsible for encoding for several subunits of these complexes. Although it is acknowledged that these changes can equally be a result of nuclear DNA malfunction, no changes to nuclear encoded mitochondrial genes have so far been found. Thus, considering the size and high informational content of the mtDNA, the lack of mtDNA repair mechanisms, and established features of mitochondrial genetics, it is not suprising that error accumulation in the mtDNA occurs with time.

1.3.1. Free radical theory of ageing:-

One of the most important factors which may contribute to mitochondrial gene mutation with age, is the high level of endogenous free radicals which are formed within the mitochondria. Harman (1972, 1981) was the first to suggest that mitochondrial DNA is a possible target of free radical attack in all tissue cells during the ageing process of the human. However, this proposal was subsequently modified by

Miquel and coworkers (1980, reviewed by Miquel 1992), who put forward the hypothesis that senescence is the byproduct of oxy-radical attack of the mitochondrial genome in fixed post mitotic cells. This modification was based upon the finding that no abnormal mitochondria could be found in fast replicating cells (Miquel et al 1979), and the observation that post mitotic cells of insects accumulate an age pigment (lipofuscin) derived from deteriorated mitochondria (Miquel et al 1978).

Especially in highly differentiated tissues, such as muscle and brain, which use high levels of oxygen, there is a release in time of amounts of oxygen radicals that may exceed the homeostatic protection of the cells. Richter et al (1988) calculated that about 1% of the oxygen consumed in the mitochondria becomes the superoxide radical and that the daily yield could reach 10^7 molecules of O_2^- per mitochondrion. Under normal physiological conditions, the superoxide radicals in the mitochondria can be destroyed by Mn^{2+} - containing superoxide dismutase (Fridovich 1988). However, the activity of this enzyme and other intramitochondrial antioxidants such as catalase and peroxidase are subject to change by various factors and may decrease with age (Reiss & Gershon 1976). As a result, reactive oxygen species (ROS) may escape detoxification, and cause deleterious effects to the mitochondrial genome (Gutteridge et al 1985).

Recent studies have shown that mitochondria of aged animals produce more ROS than those of young animals. This has been shown for both insects (Sohal 1991) and mammals (Nohl et al 1978, Sawada & Carlson 1987, Spoerri 1984). Nohl and Hegner (1978) demonstrated that more superoxide radicals are accumulated in mitochondria isolated from old rat hearts than in those from young hearts, and Sohal & Allen (1990) observed that the average rate of mitochondrial H_2O_2 release in 18 month old rats was 21% higher in the heart and 30% higher in the brain than in corresponding tissues of 3 month old rats. This age-dependent accumulation of intramitochondrial reactive oxygen species due to inadequate disposal renders mtDNA and its surrounding mitochondrial membranes more vulnerable to damage, which may prevent mtDNA replication and /or expression, and cause lipid peroxidation resulting in altered membrane permeability, posing a serious threat to organelle survival.

Evidence to support this free radical theory of ageing comes from numerous studies which have shown that the oxidized nucleoside 8-hydroxy-2'-deoxyguanosine (8OHdG) increases progressively with normal ageing in mtDNA (Ames et al 1993, Mecocci et al 1993, Hayakawa et al 1991b, 1992). These studies found that the concentrations of 8OHdG increased with normal ageing in several rat tissues (Ames et al 1993) and in mtDNA isolated from aged human brain (Mecocci et al 1993), diaphragm muscle (Hayakawa 1991b) and heart (Hayakawa 1992). Hayakawa and coworkers (1991b, 1992), found that there was a progressive increase in 8OHdG in mtDNA isolated from patients older than 65 years, where the maximal percentage increase in 8OHdG was 1.5% of the total dG in heart muscle at age 97, and 0.51% in diaphragmatic muscle at 85 years of age, in contrast to the levels found within mtDNA isolated from younger individuals which showed levels which were 25 fold lower. A high level of 8OHdG (0.87% of dG) was also observed in brain mtDNA isolated from a 90 year old individual (Mecocci et al 1993), whilst the level of 8OHdG in mtDNA of rat liver showed a 2-3 fold increase in 24 month old rats. In addition, since 8OHdG is known to cause infidelity during DNA replication (Kuchino et al 1987), the accumulation of 8OHdG in mtDNA of elderly people may have further mutational effects.

1.3.2. Changes in mtDNA structure/transcription with age:-

Numerous studies have reported structural changes in the mitochondrial DNA genome with age. In normal tissues, a minor portion of the mtDNA population, generally 2-10%, are present in the form of catenated molecules, or catenates, in which two or more circular units are interlocked as links in a chain. However, Piko et al (1977, 1984) reported a 5 to 7.5% increase in frequency of these catenates within whole brain tissue from senescent mice and rats relative to young adults. In addition to catenates, another form of complex mtDNA which occurs at a very low frequency

(about 0.1%) in normal mammalian tissues is a double sized circular molecule, or circular dimer, consisting of two monomeric genomes attached in a head-to-tail arrangement. In studies involving ageing mice (Piko et al 1977, 1984), and ageing rat tissues (Piko et al 1984), a significant increase in the frequency of these circular dimer mtDNA molecules was observed in several organs, where the frequency of circular dimers detected in senescent mouse brain and senescent rat kidney was 2% and 1.5% respectively, with smaller increases of 0.4% and 0.7% being observed within heart mtDNA from both species (Piko et al 1984). This group was also successful in identifying an age-related increase in the frequency of circular dimer mtDNA within the white blood cells of some elderly humans (Piko et al 1978).

Further analysis of these complex forms of mtDNA in senescent Balb C mice has revealed that there is a significant variability in the frequency of circular dimers in different brain regions, from 0.2% in the striatum to 3.3% in the medulla. The frequency of catenated forms also varied, where the greatest increase was observed in the cortex and the medulla compared to the other brain regions (Bulpitt & Piko 1984). However, despite the observation that these complex forms increase with age, the significance of the increased incidence of circular dimers and catenated forms in ageing tissues is as yet not understood. It is unknown whether these complex forms have any adverse consequences on mitochondrial biogenesis.

The level of mtDNA mRNA transcripts also appears to decline with an increase in age. Gadaleta et al (1990) observed that the levels of 12srRNA and the mRNA for subunit I of cytochrome c oxidase (COI) were significantly reduced in the brain and heart of senescent rats compared to their respective adult tissues. In addition, a decline in RNA products for 16SrRNA , COI and cytochrome b have also been observed during the ageing of *Drosophila melanogaster* (Calleja et al 1993), where the decrease in 16SrRNA was found to be tissue specific, with a greater reduction in the amount being present in highly energy demanding tissues such as the brain and muscle. Subsequent studies have revealed an age dependent decrease in the mtDNA content within the heart and brains of old rats (Gadaleta et al 1992, Takasawa et al 1993), and

in the tissues of old aged *Drosophila melanogaster* (Massie et al 1975), which may explain why the levels of RNA transcripts are reduced.

In addition, a previous study by Herbener (1976), reported that the number of mitochondria in the mouse heart decreases with age, with a concomitant decline in activity of the respiratory enzyme complexes I and IV. Thus, a decline in the number of mitochondria with age, resulting in a decrease in mtDNA content would result in a decrease in the level of mtDNA RNA transcripts which may explain the observed age-related decline in mitochondrial respiratory function.

1.3.3. Age related mtDNA base substitutions:-

Munscher and coworkers (1993a), were the first to report the presence of age-associated point mutations within the human mitochondrial genome. Following the examination of the extraocular muscle from 16 healthy people of different ages, using a mutation specific polymerase chain reaction method, an A-to-G transition (at 8344) mutation in the $tRNA^{Lys}$ gene was identified in a proportion of the mtDNA from 11 of these subjects (see Diagram 1.5). This point mutation has previously been reported to be a characteristic for the maternally inherited MERRF syndrome (myoclonic epilepsy with ragged red fibres) (Shoffner et al 1990), and has been shown to impair mitochondrial protein synthesis (Seibel et al 1991). No mutation was found in 5 newborns, or in two individuals younger than 20 years however, the mutation was present in two thirds of the subjects aged between 20-70 years of age and was present in all subjects tested between 74-89 years old. The amount of mutant mtDNA in comparison to the total amount of mtDNA was estimated in the extraocular muscle of a 74 and an 84 year old subject to be 2.0% and 2.4% respectively.

Allele-specific polymerase chain reaction analysis by Zhang and co workers (1993) subsequently demonstrated an A to G transition (at 3243) mutation in the $tRNA^{Leu}$ gene in a proportion of the mtDNA molecules obtained from the brain, heart,

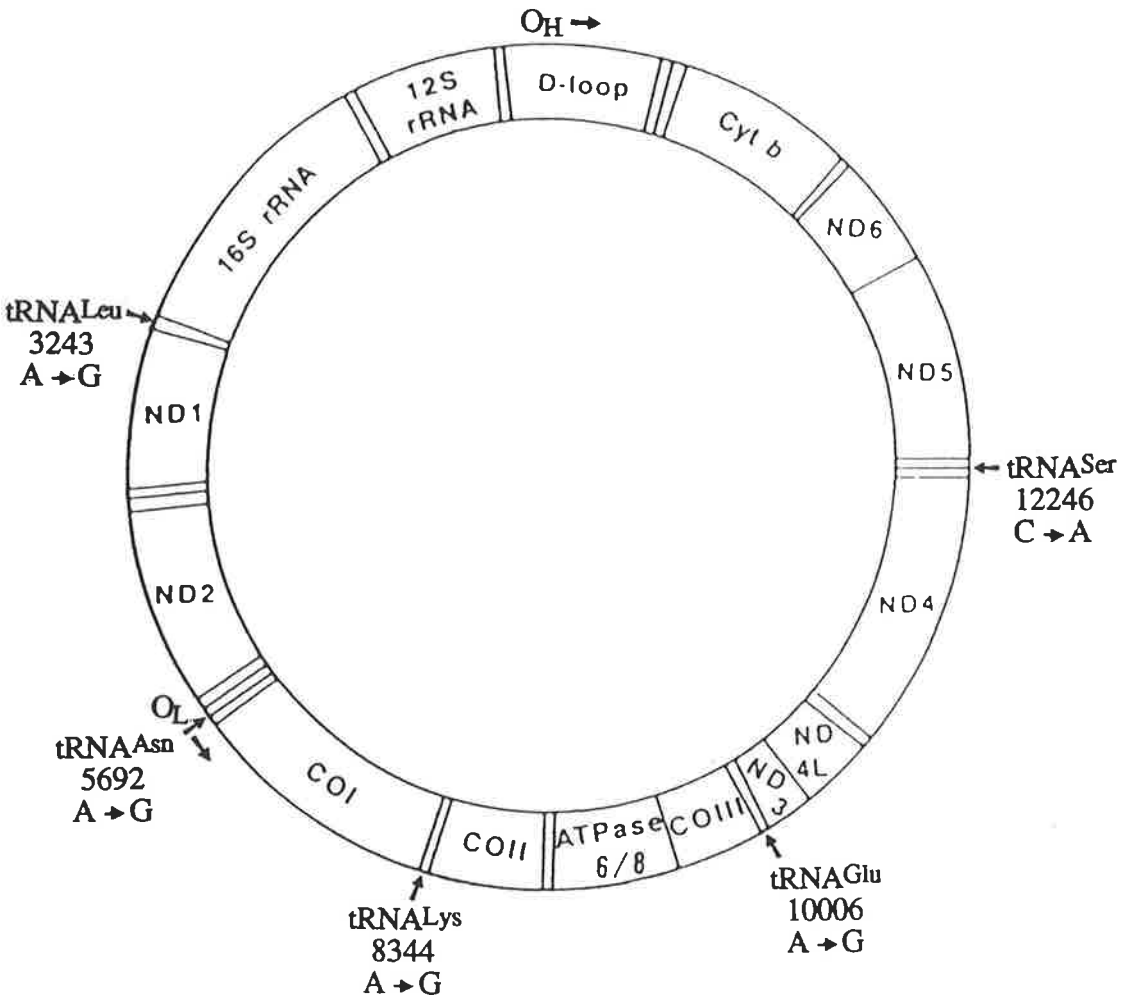


Diagram 1.5. Age-associated base substitutions within the human mitochondrial genome:-

This diagram displays the gene location of age-related mtDNA base substitutions which have been found in the human mitochondrial genome. The nucleotide positions are numbered according to the human mitochondrial genome sequence derived by Anderson et al 1981, and the base substitutions are shown where A, G and C represent the nucleotides deoxyadenosine, deoxyguanosine and deoxycytosine respectively. The open bars represent transfer RNA (tRNA) genes; 12SrRNA and 16SrRNA are ribosomal RNA genes; ND1, ND2, ND3, ND4L, ND4, ND5 and ND6 genes, encode for subunits 1, 2, 3, 4L, 4, 5 and 6 of NADH dehydrogenase (complex I); COI, COII and COIII genes encode for subunits 1, 2 and 3 of cytochrome c oxidase (complex IV); cyt b gene, encodes for cytochrome b which is a subunit of Ubiquinol-cytochrome c oxidoreductase (complex III), and ATPase 6/8 genes encode for subunits 6 and 8 of ATPase synthase (complex V). The light and heavy strand origins of replication are denoted as O_L and O_H respectively.

liver and kidneys of elderly individuals (see Diagram 1.5). This mutation has previously been reported in mitochondrially diseased patients classified as having mitochondrial encephalomyopathy, lactic acidosis and stoke-like episodes (MELAS). Oligonucleotide primers with a 3'-position specific to this mutation were designed to selectively amplify this mutant genome in preference to the wildtype genome. Seven out of 38 adult tissues tested were found to contain this mutation, whereas the mutation could not be detected in any of the 16 infant tissues analyzed. This group concluded that the appearance of this mutation in tissues of elderly individuals was age-related, whilst they acknowledged that more extensive analysis of tissues from subjects of different ages was required.

Munscher and co workers (1993b) subsequently identified the presence of multiple point mutations within the extraocular muscle of the elderly population. In addition to the previously reported 3243 and 8344 point mutations, a point mutation in the $tRNA^{Glu}$ gene (at 10006) was also identified in all subjects aged between 67-89 years old. Point mutations in the $tRNA^{Ser}$ gene (at 12246) and $tRNA^{Asn}$ gene (at 5692) were also identified however, these were less abundant, only being identified in two out of 10 tissue samples examined from different individuals of older than 67 years old. Again, these point mutated genomes were heteroplasmic, co-existing with a larger proportion of wildtype genomes.

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

Over the past four years, numerous reports have shown that during the human ageing process, various tissues accumulate mtDNA molecules which bear different size deletions (Arnheim & Cortopassi 1992). These age-associated mitochondrial DNA deletions are summarized in Table 1.2. The first indication of age-associated deleted mtDNA molecules came from a study by Ikebe et al (1990), whilst examining the brain tissue of Parkinson's diseased patients. In this report, Ikebe and co workers found that

Table 1.2. Age-associated deletions within the human mitochondrial genome:
(Refer to Diagram 1.6. for the spatial location of each of these deletions)

Deletion size (bp):	Tissues harboring the deletion:	References:
4977	Brain	Ikebe et al 1990 Cortopassi & Arnheim 1990 Ozawa et al 1990a Linnane et al 1990 Zhang et al 1992 Mann et al 1992 Cortopassi et al 1992 Soong et al 1992 Corral-Debrinski et al 1992a Blanchard et al 1993 Baumer et al 1994
	Heart	Cortopassi & Arnheim 1990 Linnane et al 1990 Corral-Debrinski et al 1991 Zhang et al 1992 Cortopassi et al 1992 Corral-Debrinski et al 1992b Baumer et al 1994
	Skeletal muscle	Linnane et al 1990 Katayama et al 1991 Cooper et al 1992 Zhang et al 1992 Cortopassi et al 1992 Simonetti et al 1992 Baumer et al 1994 Lee et al 1994
	Liver	Linnane et al 1990 Yen et al 1991, 1992, 1994 Cortopassi et al 1992 Lee et al 1994
	Ovary, uterine muscle, abdominal muscle	Kitagawa et al 1993 Suganuma et al 1993 Linnane et al 1990
	Testis	Lee et al 1994
	Others (kidney, lung, spleen skin, adrenal gland diaphragm, pancreas)	Linnane et al 1990 Cortopassi et al 1992

Deletion size (bp):	Tissues harboring the deletion:	References:
7436	Heart	Hattori et al 1991b Sugiyama et al 1991 Zhang et al 1992 Corral-Debrinski et al 1992b Hayakawa et al 1992 Hayakawa et al 1993
	Brain	Corral-Debrinski et al 1992a Linnane et al 1992 Zhang et al 1992
	Others (muscle, kidney liver, diaphragm)	Linnane et al 1992 Zhang et al 1992
3397	Diaphragm	Hayakawa et al 1991b Torii et al 1992b
2700	Diaphragm	Hayakawa et al 1991b
6063	Liver "	Yen et al 1992 Yen et al 1994
3610	Skeletal muscle	Katayama et al 1991
10423	Heart	Corral-Debrinski et al 1992b

Deletion size (bp):	Tissues harboring the deletion:	References:
5756	Muscle	Zhang et al 1992
5827	Muscle	Zhang et al 1992
6335	Muscle	Zhang et al 1992
7635	Muscle	Zhang et al 1992
7737	Muscle	Zhang et al 1992
7856	Muscle	Zhang et al 1992
8037	Heart Diaphragm Skeletal muscle	Baumer et al 1994 " "
8038	Muscle Heart	Baumer et al 1994 "
8039	Heart Skeletal muscle Muscle	Baumer et al 1994 " "
8040	Heart Muscle Skeletal muscle	Baumer et al 1994 " "

Deletion size (bp):	Tissues harboring the deletion:	References:
8041	Brain	Zhang et al 1992 Linnane et al 1992 Baumer et al 1994
	Heart	Baumer et al 1994
	Skeletal muscle	"
8042	Muscle	Linnane et al 1992
8043	Muscle	Baumer et al 1994
	Heart	"
8044	Muscle	Zhang et al 1992 Linnane et al 1992 Baumer et al 1994
8047	Heart	Baumer et al 1994
8048	Skeletal muscle	Baumer et al 1994

mtDNA molecules exhibiting a 4977bp deletion were present in the striatum of two older aged normal control subjects (64 and 73 years old). This deleted region spanned the genome from the ATPase 8 gene to the ND5 gene, consequently removing the genes for ATPase 8, ATPase 6, CO III, ND3, ND4L, ND4, ND5 and several tRNA genes (see deletion C, Diagram 1.6). This 4977bp deletion was identical to the most common deletion which has been found in the muscle mtDNA from patients affected by mitochondrial myopathies as previously discussed (Holt et al 1988, Zeviani et al 1988, Shoffner et al 1989). These age related deleted molecules were found to co-exist with wildtype mtDNA genomes, however in contrast to diseased tissues these deleted molecules in aged human tissues were by far less abundant than the wildtype genome population. As a result, in order to detect the presence of these deleted genomes in aged mtDNA samples, Polymerase Chain Reaction (PCR) analysis (Saiki et al 1988, Mullis & Faloona 1987) was required.

This PCR analysis involved the selection of PCR primers which specifically annealed to the mitochondrial genome at positions just outside of the deletion boundaries, as shown in Diagram 1.7. These primers were capable of annealing to both the wildtype and the deleted genome, however the PCR product synthesized from the deleted genome was relatively smaller than that generated from the wildtype template, due to the presence of a deletion between the two primer annealing sites. The size of the deletion within the deleted genome was then calculated by subtracting the size of the deleted PCR product synthesized from the mutant genome, away from the size of the expected PCR product generated from the wildtype genome.

At about the same time as this initial report, Cortopassi and Arnheim (1990) demonstrated that brain and heart mtDNA of normal adult tissues (>21 yrs) contained very low levels of the 4977bp deletion and that the amount of deleted mtDNA increased with age. They could not detect this deletion in fetal heart or brain tissues, which supports the original hypothesis proposed by Linnane et al (1989), that mtDNA mutations are associated with the human ageing process. Sequencing the deleted PCR product obtained, revealed that the deletion was flanked by two 13bp direct repeat

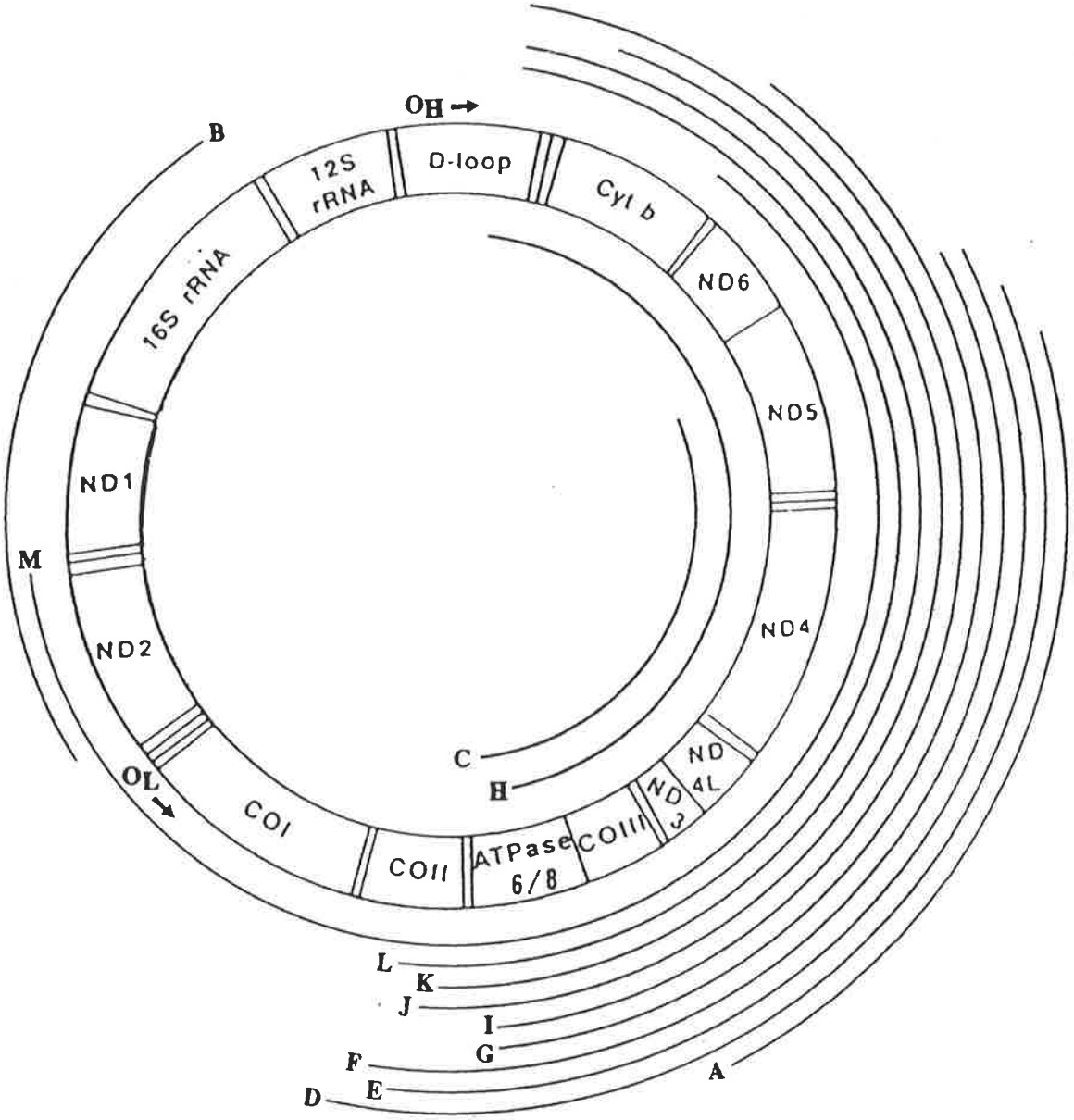
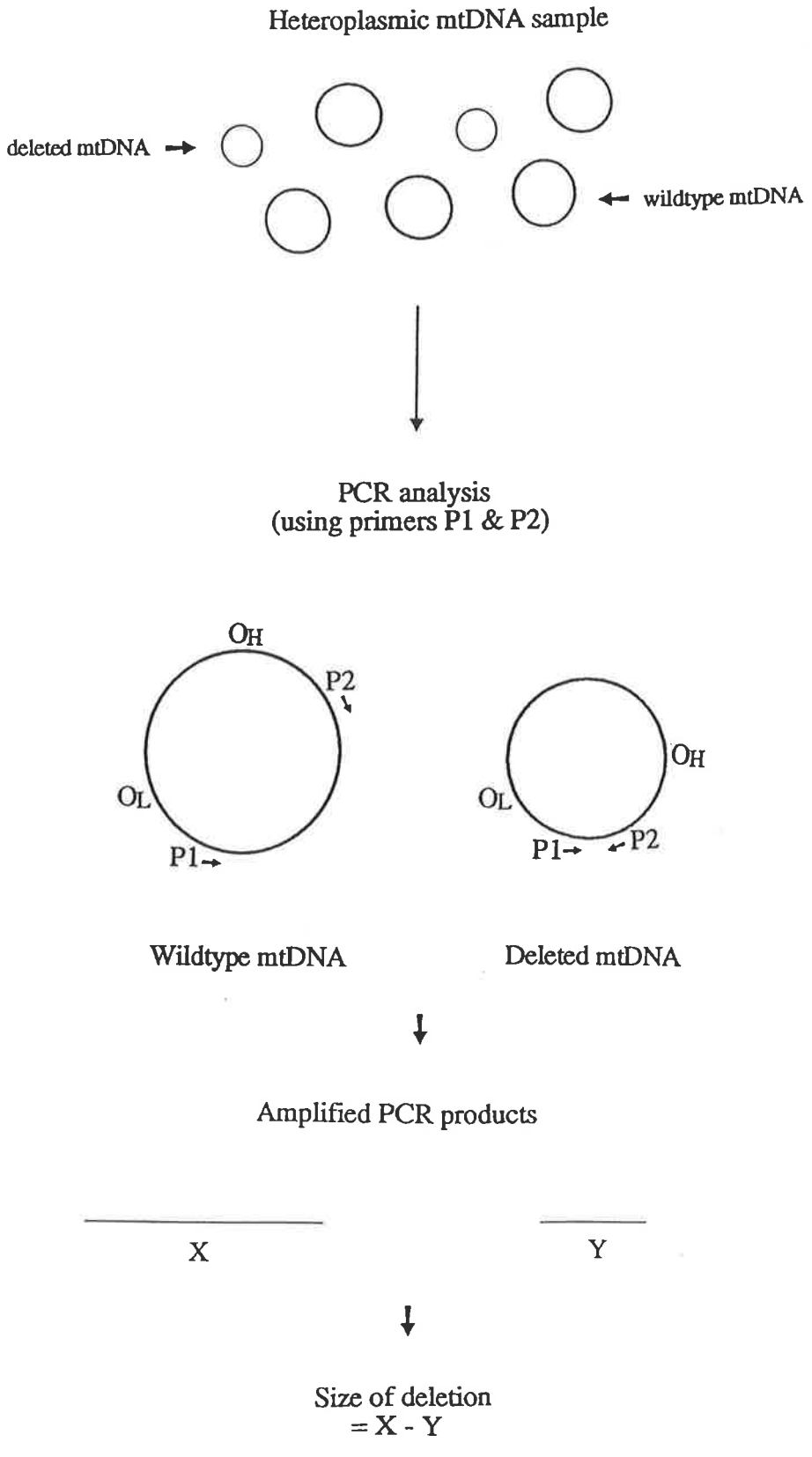


Diagram 1.6. Age-associated deletions within the human mitochondrial DNA genome:-

This diagram summarizes all the deletions which have been identified within the ageing human mitochondrial DNA genome. These deletions are numbered in alphabetical order from A to M which correspond to deletions with sizes of 3397, 3610, 4977, 5756, 5827, 6063, 6635, 7436, 7635, 7737, 7856, 8040 and 10423 basepair (bp) respectively. The most common deletions of 4977bp and 7436bp are displayed in the centre of the genetic map, whilst the remaining less common deletions are shown on the outer surface of the genome. The open bars represent transfer RNA (tRNA) genes; 12SrRNA and 16SrRNA are ribosomal RNA genes; ND1, ND2, ND3, ND4L, ND4, ND5 and ND6 genes, encode for subunits 1, 2, 3, 4L, 4, 5 and 6 of NADH dehydrogenase (complex I); COI, COII and COIII genes encode for subunits 1, 2 and 3 of cytochrome c oxidase (complex IV); cyt b gene, encodes for cytochrome b which is a subunit of Ubiquinol-cytochrome c oxidoreductase (complex III), and ATPase 6/8 genes encode for subunits 6 and 8 of ATPase synthase (complex V). The light and heavy strand origins of replication are denoted as O_L and O_H respectively.

Diagram 1.7. PCR analysis of the mitochondrial genome to detect deletions:



sequences in the human mitochondrial genome. Shortly after, Linnane et al (1990) confirmed this identical deletion in the mtDNA of multiple tissues which included the heart, brain, liver, skeletal muscle and kidney of healthy humans over 40 years old. In addition, they found that they could also detect mtDNA molecules bearing this same size deletion in the tissues of two infant tissues, by doubling the number of PCR amplification cycles to 60, which indicates the presence of very low amounts of this deleted template within the tissues of infants.

Other research groups have subsequently confirmed the presence of this most common 4977bp deleted mtDNA genome in multiple tissues which are summarized in Table 1.2, where the age of onset at which these deleted molecules were first detected varied slightly between different tissues. Within the brain, this 4977bp deletion was generally first observed in the brains of humans within their twenties (Cortopassi & Arnheim 1990, Corral-Debrinski et al 1992a) whilst within the heart (Cortopassi et al 1992, Corral-Debrinski et al 1992b) and liver (Yen et al 1991, Cortopassi et al 1992), this deletion was regularly detected in individuals which were greater than 30 years old, although subjects in their twenties have also been found to have this deleted genome in both of these tissues (Cortopassi 1990 and Lee et al 1994 respectively).

In contrast, Katayama et al (1991), and Simonetti et al (1992) independently reported the presence of mtDNA molecules harboring this 4977bp deletion in the skeletal muscle of humans which were less than a year old, whilst deleted genomes in the testis could not be detected until the sixth decade of life (Lee et al 1994). Interestingly, Kitagawa et al (1993) and Suganuma et al (1993) could only detect the presence of this 4977bp deletion in the ovarian tissue from menopausal and postmenopausal women and they suggested that this mutation may be a regulating factor of dysfunction of the ovary in ageing.

Soong et al (1992) and Corral-Debrinski et al (1992a) independently identified a mosaic pattern of distribution of this 4977bp deletion in the brains of various aged humans. Soong et al (1992) found that the level of the 4977bp mtDNA deletion varied between different brain regions with the highest levels being found in the caudate,

putamen and substantia nigra, whilst Corral-Debrinski et al (1992a) identified high levels in the putamen and the temporal cortex. Both groups found that the cerebellum contained the lowest level of these deleted mtDNA molecules.

In addition to this most common age-related 4977bp deletion, Hattori et al (1991b) reported the steady accumulation with age of a 7436bp deletion in mtDNA obtained from aged human hearts that were over 30 years old. Sequencing the obtained deleted PCR product revealed that this deletion spanned the genome from the ATPase6 gene into the D loop region, and was surrounded by a 12bp direct repeat sequence (see deletion H, Diagram 1.6). Further studies confirmed the presence of this age associated 7436bp deletion which appeared to be specific to the heart muscle tissue where the level of molecules harboring this deletion increased with age (Corral-Debrinski et al 1992b, Sugiyama et al 1991, Zhang et al 1992, Hayakawa 1992, 1993). However, analysis of brain tissue subsequently revealed that this size deletion was also present within the brains of humans who were greater than 67 years of age (Corral -Debrinski 1992a), and Linnane et al (1992) also revealed that this 7.4Kb deletion was present in multiple tissues (eg. heart, brain, muscle, liver and kidney).

Analysis of diaphragm muscle (Hayakawa et al 1991b) was one of the first reports that revealed that more than one size deleted mtDNA molecule could be present within the same mtDNA sample obtained from aged individuals. This study identified two deletions which were relatively smaller than the most common deletions previously discussed, and consisted of a 3397bp deletion and a 2700bp deletion which were present within diaphragm muscle samples obtained from humans aged 55 years or older. Torii et al (1992b) subsequently sequenced the deleted PCR product obtained from the 3397bp deletion, and discovered that this deletion was flanked by two 7bp direct repeat sequences which were located within the CO III gene and the ND5 gene respectively. Thus, this deletion was found to remove the genes encoding for ND3, ND4L, ND4, ND5, CO III, and five tRNA genes (see deletion A, Diagram 1.6). The incidence of these deleted molecules exponentially increased with age where 100% of subjects over 80 years of age were found to house this deleted genome. In addition,

this group (Torii et al 1992b) also discovered other deleted PCR products which may represent potential deleted mtDNA templates within the samples analyzed however, these products were not fully characterized to determine if they were genuine deleted PCR products.

In addition to precisely defining the most common deletion of 4977bp in the ageing liver (Yen et al 1991), Yen and coworkers (1992) revealed that a larger size deletion of 6063bp was also present within the mitochondrial genomes of human livers which were over 40 years old. Restriction digestion and sequencing of the deleted PCR product obtained from templates housing this deletion revealed that the endpoints of the deletion were flanked by an imperfect 8bp direct repeat sequence, which were positioned within the COX II gene and the ND5 gene, resulting in the removal of the genes encoding for ATPase 6 and 8, COX III, ND3, ND4L, ND4 and partially removing the COX II and ND5 genes (see deletion F, Diagram 1.6). Subsequent analysis revealed that molecules harboring either the 6063 deletion or the 4977bp deletion were capable of co-existing within the same liver mtDNA sample (Yen et al 1992, 1994), where the deleted PCR product amplified from the 4977bp deleted template was represented by a much brighter band within the agarose gel than that of the 6063bp deleted molecule, which indicates that the 4977bp deleted mtDNA is more abundant than the 6063bp deleted mtDNA in the mitochondria of human liver during the ageing process.

Using different oligonucleotide primer pair combinations for PCR, Zhang and co workers (1992) detected multiple deletions in several tissues of a 69 year old female subject. Apart from the most common deletions of 4977bp and 7436bp, eight additional deletions with sizes of 5756, 5827, 6335, 7635, 7737, 7856, 8041 and 8044bp were identified (see deletions C, H, D, E, G, I, J, K, L, Diagram 1.6 respectively). All of these were found to occur between short direct repeats ranging from 5 to 13 bp in size. This study demonstrated the fact that a given pair of PCR primers do not necessarily reveal all the deletions between PCR priming sites, but rather result in the preferential amplification of the mtDNA segments with the longest deletions (shortest PCR

products). Thus, Zhang and colleagues (1992) proposed that the total extent of mtDNA deletions in any single cell or tissue may have previously been seriously underestimated.

This group further identified multiple deletions in the muscle, heart, brain and skeletal muscle of aged humans, which all involved the presence of the same 5bp direct repeat which had sequences positioned in the COX II gene and in the D-loop region (Baumer et al 1994). These deletions had sizes of 8037, 8038, 8039, 8040, 8041, 8043, 8044, 8047, 8048 which only differ by a single base pair. In addition, Linnane et al (1992), reported a deletion of 8042bp involving the same direct repeat, and confirmed the presence of the 8041bp and 8044bp deletions in the ageing human mitochondrial genome in various tissues. This group of deletions is known collectively as the 8.04Kb deletion family which result in the removal of the genes encoding for COX II, ATPase 6 and 8, COX III, ND3, ND4L, ND4, ND5, ND6, Cyt.b, partially removing the D-loop region and several tRNA genes (see deletion L, Diagram 1.6).

So far, all the previously described age-related deletions have been found to exist within the major region between the two origins of replication, resulting in the removal of major structural genes of cytochrome c oxidase (COII, COIII), ATPase Synthase (ATPase 6, 8), and NADH dehydrogenase (ND3, 4, 4L, 5, 6). These deletions are in general located in the same regions as those deletions that have been associated with patients with ocular myopathies (Mita et al 1990, Degoul et al 1991). However, Katayama and co workers (1991) were successful in identifying an age-dependent 3610 bp deletion which was present within the minor region between the two origins of replication spanning the genome from the 16SrRNA gene to the ND2 gene (see deletion B, Diagram 1.6). This deletion was detected within skeletal muscle samples obtained from subjects of over 60 years old. Sequencing the deleted PCR product generated from this deleted template revealed that the deletion was flanked by a four base pair direct repeat sequence. So far, this is the only age-associated deletion that has been found to excise the ND1 gene and part of the 16S rRNA gene.

A common feature of the ageing mitochondrial DNA deletions is that they all seem to spare both of the origins of replication. This is not suprising since a molecule bearing a deletion of either replication origin would not be capable of successfully replicating itself, which would inhibit this mutant genome from accumulating with age. However, a 10423 bp deletion was identified within the ageing human heart by Corral-Debrinski et al (1992b), which results in the removal of the origin of replication of the light strand of the genome. Sequence analysis of this region revealed that the deletion is flanked by a 10 bp direct repeat sequence resulting in the removal of the genes encoding for ND2, COX I, COX II, ATPase 6 and 8, COX III, ND3, ND4L, ND4, ND5, ND6, cytochrome b and several tRNA genes (see deletion M, Diagram 1.6). This deletion was found in subjects that were greater than 40 years of age and is identical to the deletion which has previously been found within the genomes of patients with diabetes mellitus (Ballinger et al 1992).

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

Deleted mtDNA molecules within aged human tissues have been exclusively detected by PCR analysis, since the levels of these deleted genomes which accumulate with age, including the most common and abundant 4977bp deletion, are too low to be detected by Southern hybridization techniques which have been utilized for detecting the presence of these molecules in the tissues of diseased patients. PCR analysis has revealed that tissues such as the brain, heart and skeletal muscle have the highest levels of deleted mtDNA while most other tissues have significantly lower levels. So far, attempts at detecting age-associated deleted mitochondrial DNA genomes in proliferative cells such as those found in blood have failed (Yen et al 1991, 1992, Lee et al 1994).

Initially, researchers in this field of study provided qualitative information concerning the identity and tissue specificity of the deletions in mtDNA. Cortopassi and

Arnheim (1990), using a semi quantitative dilution PCR method provided the first rough estimation that about 0.1% of the total mtDNA population in brain tissue of middle aged adults, contained the 4977bp deletion (see Table 1.3). These results were confirmed by Blanchard et al (1993), and Ozawa et al (1990a) also reported similar levels by developing a quantitative method based on kinetic PCR analysis. In contrast, Mann et al (1992) calculated a 10 fold lower level ranging from 0.01-0.02% in subjects ranging from 72 -86 years old by applying a different quantitative method which involved constructing a standard curve of the amount of deleted PCR product synthesized relative to the initial amount of deleted mtDNA in the reaction mixture.

Soong et al (1992) and Corral-Debrinski (1992a) went on further to identify a mosaic pattern of distribution of this deletion within the brains of humans. Using the standard curve PCR method, Soong et al (1992) found that the substantia nigra, caudate and putamen had the highest levels of this deleted genome (0.46%, 0.25%, and 0.012% in an 84 year old subject respectively), whilst Corral-Debrinski (1992a) calculated higher levels of 12% and 3.4% in the putamen and temporal cortex respectively of an 80 year old subject using the semi quantitative dilution PCR method.

Quantitation of the level of this most common deletion within other tissues has yielded varying results (see Table 1.3). Within the heart and skeletal muscle similar levels were calculated which ranged from 0.0005 to 0.1% in the heart using the dilution PCR method (Corral-Debrinski 1991, 1992b, Cortopassi & Arnheim 1990) and 0.001-0.1% in skeletal muscle using the standard curve PCR method (Cooper et al 1992, Simonetti et al 1992), whilst in the liver a lower level of 0.007% was quantitated by the dilution PCR method (Lee et al 1994). In contrast, much higher levels were reported in abdominal muscle samples where a value of 6.5% was calculated in a 50 year old female subject by applying the kinetic PCR reaction (Kitagawa et al 1993). The ovarian tissue of this same subject also had a high level of genomes bearing this deletion of 4.5%, which is approximately 90x greater than the levels found in the testis of men aged between 80 and 89 years of age which were calculated by the dilution PCR method (Lee et al 1994).

Table 1.3. Quantitative analysis of age associated deleted mtDNA molecules within human tissues: This table summarizes the level of deleted mtDNA molecules which have been calculated in aged human tissues by various research groups. The level is expressed as the percentage (%) of deleted mtDNA genomes present relative to the total amount of mtDNA within each tissue.

Deletion size (bp):	Tissue:	% deleted mtDNA:	References:
4977	Brain		
	middle age	0.1%	Cortopassi et al 1990
	38 years	0.3%	Ozawa et al 1990a
	72-86 years	(0.01-0.02%)	Mann et al 1992
	>80 years		
	(putamen)	12%	Corral-Debrinski 1992a
	(temporal cortex)	3.4%	"
	86 years		
	(cerebellum)	0.0067%	"
	82 years		
	(substantia nigra)	0.46%	Soong et al 1992
	(caudate)	0.25%	
	(putamen)	0.012%	
	neonates		
	(all regions)	0.0004%	"
	71-83 years	0.12%	Blanchard et al 1993
	(frontal cortex)		
	Heart		
	middle aged	0.1%	Cortopassi et al 1990
	78 years	0.0035%	Corral-Debrinski 1991
	>60 years	0.0005-0.007%	Corral-Debrinski 1992b
	Skeletal muscle		
	21 years	0.001%	Cooper et al 1992
	56 years	0.01%	"
	78 years	0.02%	"
	84 years	0.1%	Simonetti et al 1992
	70-79 years	0.06%	Lee et al 1994

Deletion size (bp):	Tissue:	% deleted mtDNA:	References:
4977	Liver		
	70-79 years	0.007%	Lee et al 1994
	Abdominal muscle		
	36 years	3%	Kitagawa et al 1993
	50years	6.5%	"
	ovary		
	36 years	0.04%	Kitagawa et al 1993
	50 years	4.5%	"
	Testis		
	80-89 years	0.053%	Lee et al 1994
7436	Heart		
	80 years	3%	Sugiyama et al 1991
	90 years	9%	"
	97 years	7%	Hayakawa et al 1992
	80 years	0.92%	Hayakawa et al 1993
	90 years	2.55%	"

In addition to the most common age-related deletion, several groups have also attempted to quantitate the level of the 7436bp deletion within ageing cardiac muscle using the kinetic PCR quantitation method. These studies revealed levels which ranged from 0.92% in an 80 year old individual, to 9% in a subject that was 90 years old (Hayakawa et al 1992, 1993, Sugiyama et al 1991). In general, the levels of this 7436bp deletion in the heart were approximately 90x higher than the level of the 4977bp deleted molecules which were found to exist in cardiac tissue, which indicates that mitochondrial genomes within the ageing heart are more likely to acquire a 7436bp deletion.

Combining the data accumulated in the past few years, the proportion of deleted mtDNA molecules calculated within the same tissue and between tissues seems to vary, which is most likely due to the different quantitation methods used. However, despite this variation, the levels are relatively low. Since there are 2-10 copies of mtDNA in one mitochondrion, and numerous mitochondria per cell, the pathological consequences of the existence of such low levels of deleted mtDNA in human tissues remains the topic of much debate. Cooper et al (1992) suggested that the presence of the 4977bp deletion in skeletal muscle was not present at high enough levels to alone be having a detrimental effect on an organism, and they postulated that this deletion may be just the tip of the mutational iceberg, representing a mere fraction of the total genetic damage to the mitochondria with age.

In comparison to the levels found in the mitochondrial diseased patients previously discussed, which range from 20-90% of the total mtDNA population, the proportion of deleted molecules in aged tissues are between 1000-10 000 fold lower. In the skeletal muscle of patients with KSS, the presence of such high levels of deleted mtDNA have been found to have a dominant physiological effect (Shoubridge et al 1990), however, the threshold level of the mutant/wildtype ratio necessary for deleted mtDNA to produce such affects is unknown. Tissues such as the heart, skeletal muscle and brain have particularly high demands for oxidative energy, and hence are likely to have relatively low thresholds for mitochondrial dysfunction. Therefore, if deleted

mtDNA levels reached such a threshold level in these particular tissues during the normal course of ageing in an individuals life, pathological effects similar to those seen in KSS could arise.

Recent experiments by Hayashi and coworkers (1991) involving the introduction of disease related deleted mtDNA into HeLa EB8 cells which lack mtDNA, has shown to cause mitochondrial dysfunction in isolated cybrid clones. Following quantitation of the deleted mtDNA in these cybrids, they found that deleted mtDNA which had accumulated to over 60% of the total mtDNA resulted in a progressive inhibition of overall mitochondrial translation, as well as a reduction of COX activity. Thus, it seems that the levels of deleted mtDNA within muscle may have to reach levels of greater than 60% to have any severe impact on the bioenergetics of the cell.

1.3.6. Age-associated mtDNA deletions in other species:-

Early electron microscopic data by Piko et al (1988) suggested the possibility that mtDNA from senescent mouse liver might contain small deletions and / or insertions, since duplex molecules with single-stranded loops were formed when aged mtDNA samples were heat denatured and subsequently reannealed by cooling. The incidence of these heteroduplexes was found to increase from 1% in young mouse liver, to 5% of the mtDNA in adult mouse liver.

A subsequent study involving PCR analysis has revealed that an age-related 4834 bp mtDNA deletion accumulates within the liver and brain of adult and senescent rats (Gadaleta et al 1992). The proportion of deleted molecules in the liver was estimated to represent 0.02% and 0.0005% of the total mtDNA in senescent and adult rat liver respectively. Closer examination of this 4,834 bp deletion revealed that it was flanked by a 16bp direct repeat sequence positioned in the ATPase 6 gene (at 8103) and the ND5 gene (at 12937).

Multiple mitochondrial DNA deletions have also been associated with the ageing process of the Rhesus monkey (Lee et al 1993). PCR analysis of skeletal muscle mtDNA from various aged (6-27 years) monkeys revealed that a proportion of their genomes harbored deletions. Seven different deletions ranging from 5.4Kb to 8.1Kb were detected in a 27 year old animal whilst none were identified in several 6 and 7 year olds. Sequence analysis of a 8Kb deletion revealed that this deletion was flanked by a 5 bp direct repeat sequence.

Deleted mtDNA molecules were also found to accumulate in an ageing population of nematodes, *Caenorhabditis elegans* (Melov et al 1994). Via PCR, eight deletions were identified, where four of these were examined and were found to be surrounded by direct repeat sequences of 4-8bp. Deletions of the mitochondrial genome were also found to be associated with the process of senescence in the filamentous fungus *Podospora anserina* (Sainsard-Chanet et al 1993). In contrast, a deletion within the mouse mitochondrial genome was identified in two wild mice (Boursot et al 1987), however, closer analysis of this deletion failed to show any age-dependent relationship (Nelson et al 1993).

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

The most widely discussed special feature of these deletions in the ageing mitochondrial genome is that the majority of these are flanked by tandem direct repeat sequences. This feature may hold the answers as to how these deletions are formed within the genome. Table 1.4, shows the nucleotide sequences which flank the breakpoints of all the reported age associated deletions within the human mitochondrial genome. These deletions can be divided into two classes. The most commonly observed deletions usually fit the criteria for class I deletions, where precisely one copy of the repeat is retained in the subgenomic (deleted) molecules, whilst the other is removed with the deleted fragment. However, in class II the deletion endpoints are

Table 1.4. Direct repeat sequences flanking age-related deletions within the human mitochondrial DNA genome:

This table summarizes the sequences which surround age-associated deletions within the human mitochondrial genome.The fragments deleted from the genome are bounded in brackets ( ) and the repeat sequences are underlined. Class I deletions are surrounded by tandem direct repeat sequences where exactly one repeat is retained while the other is deleted, in contrast to class II deletions which are either flanked by imperfect repeat sequences or have breakpoints which are located in the middle of the repeat sequences such that exactly one full repeat sequence is not retained within the deleted genome.

Deletion size (bp):	Nucleotide sequence flanking the deletion breakpoints:
Class I:	
4977	8470 ACCTCCCTCACCA (.....13447 ACCTCCCTCACCA) ATPase 8 ND5
7436	8637 CATCAACAACCG (.....16073 CATCAACAACCG) ATPase 6 D-loop
3397	9568 TCACCCC (.....12965 TCACCCC) COX III ND5
3610	1837 CCCC (.....5447 CCCC) 16S rRNA ND2
5756	7769 GAAACCG (.....13525 GAAACCG) COX II ND5
5827	7954 TCCCCC (.....13781 TCCCCC) COX II ND5

Deletion size (bp):	Nucleotide sequence flanking the deletion breakpoints:
6335	8470 ACCTCCC (.....ACCTCCC) 14805 ATPase 8 Cyt. b
7635	8433 TCACCCA (.....TCACCCA) 16068 ATPase 8 D-loop
7737	7986 GACTCCT (.....GACTCCT) 15723 COX II Cyt. b
8041	8030 CCCAT (.....CCCAT) 16071 COX II D-loop
10423	4389 CACCCCATCC (.....CACCCCATCC) 14812 $tRNA^{Ile}$ Cyt. b

Class 2:

6063	7842 TAACAGAC (.....CAACATAC) 13905 COX II ND5
7856	8207 CCCATC G (.....) A CCCATC 16071 COX II D-loop

Deletion size (bp):	Nucleotide sequence flanking the deletion breakpoints:
2700	uncharacterized
8037	8030 CCCA (T.....) CCCAT 16071 COX II D-loop
8038	8030 CCCA (T.....C) CCAT 16071 COX II D-loop
8039	8030 CCC (AT.....C) CCAT 16071 COX II D-loop
8040	8030 CCC (AT.....CC) CAT 16071 COX II D-loop
8043	8030 CC (CAT.....CCCA) T 16071 COX II D-loop
8044	8030 C (CCAT.....CCCA) T 16071 COX II D-loop
8047	8030 C (CCAT.....CCCAT CA) 16071 COX II D-loop

surrounded either by an imperfect direct repeat where one sequence is retained, or the deletion breakpoints are positioned in the middle of each repeat sequence such that exactly one full repeat sequence is not retained within the deleted genome. Baumer et al (1994) found that multiple deletions of approximately 8.04Kb existed at the same repeat site, which only differed by a single base pair, where the subgenomic molecules retain >1 copy, 1 copy, or <1 copy of the 5 base pair repeat.

It therefore seems likely that more than one mechanism is operating in causing these age dependent mtDNA deletions. According to Mita et al (1990), the phenomenon in humans may be caused by a homologous recombinational event in the region bordered by the repeat sequences, via slipped mispairing. From analyzing the 13bp direct repeat which surrounds the most common human deletion, Mita et al (1990) observed the presence of two AT rich sequences, 13bp and 20bp long respectively, flanking both sides of the ATPase 8 gene direct repeat. These AT-rich sequences have the potential to form bent DNA and putative bent DNA regions may form a triplex helix with a displaced single-stranded loop called H-DNA (Mirkin et al 1987, Htun & Dahlberg 1988), thus favoring a slipped mispairing recombinational event.

Shoffner and coworkers (1989) also put forward the theory that these deletions may be formed via strand slipped mispairing during the replication of the mitochondrial DNA molecule, since the heavy strand is exposed as a single strand for a considerable time during replication. Thus, this would imply that deletions may occur randomly in all regions of the genome that contain direct repeat sequences. Due to the large number of direct repeat sequences of 4bp or greater, this would provide an abundance of potential sites for deletions, and as a result, a broad spectrum of deletions could accumulate in the human mtDNA genome during ageing.

The finding that tissues with the highest levels of deleted mtDNA molecules are the most aerobically active, lead researchers to propose the possible involvement of oxygen free radicals in the formation of these deletions. High oxygen consumption is expected to be correlated with high levels of oxidative DNA damage due to the large amount of oxygen radicals generated from the electron transport chain (Forman &

Boveris 1982, Boveris & Cadenas 1982), which can lead to base adduct formation and single strand breaks. Arnheim (1992) postulated that the formation of adducts on the bases may promote deletion formation during DNA replication in vivo.

Hayakawa and coworkers (1991b, 1992, 1993) were among the first to provide evidence that such a relationship exists between oxygen radicals and mtDNA deletions. They reported that the level of the oxidized base 8OHdG in diaphragm and heart mtDNA increased with age. Concomitant with this increase, they also observed that the proportion of deleted genomes bearing the 4977bp deletion (Hayakawa et al 1991b) and the 7436bp deletion (Hayakawa et al 1992, 1993), also increased thereby suggesting the possible involvement of oxygen radicals in the formation of mitochondrial DNA deletions.

In addition, Corral-Debrinski et al (1991b) quantitated the level of the 4977bp deletion in the genomes of normal human hearts in comparison to the levels found in ischemic hearts. Ischemia has been reported to result in an increase in oxygen radical generation (Arroyo et al 1987, Otani et al 1984). This study found that much higher levels of 0.02%-0.85% deleted mtDNA with respect to the total mtDNA population were found in ischemic hearts compared to 0.0035%, which was the maximum level reached in a normal control 78 year old individual. Again, this higher level of deleted mtDNA molecules observed under oxidative stress highlights the possible involvement of free radicals in the formation of deletions.

The involvement of oxygen radicals is further supported by the work documented by Soong et al (1992), where they found a regional variability in the distribution of 4977bp-deleted genomes within the brain. They found the highest levels were present in the substantia nigra, caudate and putamen which are sites characterized by high dopamine metabolism which produces hydrogen peroxide as a byproduct. Hydrogen peroxide is a powerful oxidant that can lead to the production of oxygen radicals, hence, Soong and colleagues suggested that somatic deletions may be produced by oxidative damage. In addition, Yen and coworkers (1994) recently revealed that concomitant with the increase in presence of deleted mtDNA molecules

with age, there is also an increase in the levels of hepatic lipid peroxides (malondialdehyde, MDA), and manganese superoxide dismutase (Mn-SOD) activity which is a scavenger of superoxide radicals. These authors suggested that the increased Mn-SOD maybe synthesized to cope with the increased oxygen free radicals in tissue cells in senescence, and further proposed that the enhanced generation of lipid peroxides in mitochondria during the ageing process may damage mtDNA leading to the formation of deletions.

Finally, the role of oxidants in the formation of mtDNA deletions is supported by the observation that doxorubicin, a compound that stimulates mitochondrial oxidant production, results in the formation of deletions within the mtDNA of cardiac tissue (Adachi et al 1993), where this effect is blocked by ubiquinone (Coenzyme Q_{10}), a key component of the mitochondrial electron transport system whose reduced form, ubiquinol, exhibits antioxidant properties (Frei et al 1990).

However, whilst these above theories suggest possible mechanisms of deletion formation, at this stage, the exact underlying mechanism of deletion formation within the mitochondrial genome remains to be determined. At present it is not possible to distinguish whether a deletion event occurs only once in a tissue early in development, followed by exponential accumulation of progeny of that molecule, or from a situation in which deleted mtDNA are continuously generated throughout an individual's lifespan with each de novo deleted mtDNA accumulating at its own exponential rate.

1.3.8. Consequences of the accumulation of deleted mtDNA molecules with age:-

Due to the limited DNA repair mechanisms operating within the mitochondria, it is not suprising that these deleted mtDNA molecules accumulate with time. When a deleted mtDNA molecule arises within a cell, it would be replicated alongside the normal wildtype genomes. Shoffner et al (1989) suggested that since all the enzymes and factors required for mtDNA replication are encoded by the nuclear genome, the

deleted molecules being smaller would be replicated faster, resulting in an accumulation of these deleted genomes with age. Cortopassi & Arnheim (1990) also suggested that these deleted genomes may have an intracellular advantage since sequences involved in down-regulating mtDNA replication may be lost in the deletion event.

Upon cell division, where mtDNA molecules are randomly distributed, differential loads of mutant mtDNA would partition into progeny cells within given tissues or organs. Tissues dependent on ATP production would continue to function adequately until the level of deleted genomes reached a threshold level where the synthesis of mitochondrial encoded subunits was insufficient to assemble with the nuclear encoded subunits, resulting in an impairment of the assembly of the respiratory chain complexes. Thus, cells that have a high demand for energy will be particularly sensitive to the accumulation of deleted mtDNA molecules, and consequently may show a disturbance even if a small number of mtDNA are mutated. It is proposed that eventually the cellular energy production will decline below the levels required to reach cellular energy demands, thereby posing a threat to cell viability.

Histochemical methods have recently revealed a mosaic pattern of loss of cytochrome c oxidase activity as a function of age in human cardiomyocytes (Muller-Hocker 1989), skeletal and diaphragm muscle (Muller-Hocker 1990, Byrne & Dennet 1992) and in extraocular muscle (Muller-Hocker et al 1992, 1993). These studies found that the loss of enzyme activity occurred sporadically and the proportion of effected cells or fibres increased with age. Irrespective of the number of defects, the enzyme deficiency always affected isolated cardiomyocytes, or muscles fibres ending abruptly at the edge of neighboring cells or fibres. Interestingly, this deficiency of cytochrome-c-oxidase is also a characteristic finding of patients with mitochondrial myopathies as previously discussed. Analysis of individual cytochrome c oxidase deficient muscle fibres in patients with mitochondrial diseases has revealed that transcripts of deleted mtDNA molecules are more abundant (Oldfors et al 1992, Prelle et al 1994, Collins et al 1991, Mita et al 1989), and Shoubridge et al (1990) and Collins et al (1991) suggested that these deleted molecules may be acting in a dominant fashion to cause this

enzyme deficiency. Recently, Muller-Hocker et al (1993) tried to determine whether deleted molecules are also more abundant within the cytochrome c oxidase deficient muscle fibres of elderly patients however, their results displayed variability where normal, reduced or negative signals were observed. Therefore, further analysis is required to determine if these deleted mtDNA molecules are responsible for the observed decline in cytochrome c oxidase activity seen in the elderly population.

The removal of a segment of the genome also leaves behind a hybrid gene which is a combination of the two genes which originally surrounded the deleted fragment. Sequencing the remaining genome after the 7436bp deletion was removed within heart mtDNA, Hattori et al (1991) reported the presence of a stop codon which is not present within the wildtype genome. This stop codon would cause premature termination of transcription, resulting in the synthesis of a shorter abnormal protein, which might disturb the molecular assembly of the energy transducing complexes. As a result, this would lead to the incomplete reduction of molecular oxygen with a concomitant increase in oxygen free radical production, causing further destruction to the genome and its surrounding membranes.

One suggestion of the fate of the deleted mtDNA fragments which are removed from the mitochondrial genome, is that they may escape from the mitochondria and they may be translocated into the cytoplasm (Wei 1992). It has been proposed that these transposed DNA fragments, if not degraded, may be able to insert into the nuclear DNA. Reid (1983) further proposed that migrating mtDNA molecules when inserting into the nuclear genome, may cause activation of certain oncogenes. MtDNA fragments have been detected in the genomic DNA of human placenta, leukocytes and several human cell lines (Kamimura et al 1989). Recently, the c-myc oncogene in HeLaTG cells was found to contain the sequence encoding for cytochrome c oxidase subunit III (Shay et al 1991), which suggests a possible involvement in the carcinogenic process. Other reports (Gellissen et al 1983, Hadler et al 1983) have also indicated that mtDNA fragments, generated from incomplete degradation of deleted DNA or during the normal cellular autophagic process, may act like mobile genetic elements, eventually becoming

inserted into the nuclear genome. The mechanism by which the mtDNA fragments are inserted into the nuclear DNA is unclear, however, Thorsness and Fox (1990) established that migration of cytoplasmic genetic elements into the nucleus can occur at a very high frequency. Therefore, it has been suggested that mutations and deletions in the mtDNA may be involved in some carcinogenic processes (Shay et al 1987, 1992, Yamamoto et al 1992, Welter et al 1989).

In conclusion, it is proposed that deletions progressively accumulate throughout life and that the occurrence of these deleted mtDNA molecules in individual tissues may limit the ability of the tissue to function normally. It is widely acknowledged that the accumulation of deleted mtDNA molecules may only reflect a small proportion of the total mtDNA mutations which occur with an increase in age. However, combining all the possible mtDNA mutations (deletions / insertions, point mutations, control of transcription / translation), the total accumulation of aberrant mtDNA with age may contribute significantly to the observed decline in respiratory function and play a major role in the ageing process. Therefore, ageing may be the most widespread mitochondrial disease of all.

1.4.0. Objectives of this study:-

Although recent studies have shown that several species accumulate deleted mtDNA molecules with age, prior to commencing the project described in this thesis, research examining the relationship between ageing and the accumulation of deleted mtDNA molecules was confined to the human genome. There was no report within the literature concerning the appearance of any such deletions accumulating in the ageing mitochondrial genomes of other species, except for the previously described study by Piko et al (1988) which suggested that deletions and/ or insertion may exist within the

ageing mouse genome. Therefore, the overall aim of this study was to determine whether the accumulation of deleted mtDNA molecules, during the normal ageing process, was confined to the human or was a phenomenon also shared by other species.

The ageing mouse was selected as an experimental model, since the mouse mitochondrial genome has been completely sequenced and the overall order of gene organization is identical to that of the human (Bibb et al 1981, Anderson et al 1981). The first objective of this study was to develop a PCR method which was specific to the mouse mitochondrial genome, in order to detect the presence of deleted mtDNA molecules. Due to the unknown size, number and position of deletions which may accumulate within the ageing mouse genome, this involved designing a complex PCR scheme, involving multiple primer pair combinations to methodically scan the mouse mtDNA genome for deletions. This search was limited to the major region spanned by the two replication origins, since the majority of human age-related deletions have been confined to this area. The second objective was to then use this PCR method to examine the brain, heart, skeletal muscle and liver of various aged mice for the presence of deleted mitochondrial DNA molecules.

Chapter 2:

Materials and Methods

2.0. Experimental animals:-

All experiments were performed on tissues obtained from female *Balb C* mice that were 10 weeks old, 19 months old and 42 months old respectively (Table 2.1). These mice were housed in rooms maintained at a constant temperature of 25°C, under a 12hr light / dark cycle. All the mice were fed on standard colony diet which was supplied *ad libitum*, except for the 42 month old mice which were obtained from a previous dietary restriction study where their average food intake of *ad libitum* feeding was reduced by 40%. Each animal was healthy at the time of sacrifice, except for two 42 month old mice which upon dissection were found to have enlarged spleens. The mice were initially anaesthetized with ether and subsequently sacrificed by cervical dislocation. After dissection, the following tissues were removed from each animal; brain, heart, skeletal muscle and liver. Following removal, each tissue was snap frozen in liquid nitrogen and was subsequently stored at -80°C until the mitochondrial DNA could be isolated and analyzed. This protocol was approved by the Animal Care and Ethics Committee at the CSIRO - Division of Human Nutrition, Adelaide, Australia (Ethics #299).

Table 2.1. Female *Balb C* mice tissues analyzed:

	Brain:	Heart:	Muscle:	Liver:
10 weeks old	N=10	N=10	N=10	N=10
19 months old	N=5	NA	NA	N=5
42 months old	N=9	N=9	N=9	N=9

NA = Tissues not available due to -80°C freezer malfunction.

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

2.1.1. Isolation of mitochondria:

Mitochondrial preparations were obtained from homogenized *Balb C* mice tissues by differential centrifugation. The procedure employed was a modification of that described by Hauswirth et al (1987), for the small scale preparation of animal mitochondria. After thawing at room temperature, the tissues were placed in sterile petri dishes on ice which contained approximately 2ml of homogenizing buffer (0.34M sucrose, 1mM EDTA, 10mM Tris-HCl, pH7.5), where they were finely minced with sterile scissors. Following the removal of the surrounding buffer in each dish, each sample was resuspended in 5ml of the same homogenizing buffer and was subsequently homogenized with an Ultra-Turrax homogenizer (Janke & Kunkel IKA-Labortechnik) for 30 seconds on medium/high speed. Nuclei and cellular debri were removed by two successive low speed centrifugations at 1000 x g for 10 minutes at 4°C, using a Beckman J6 centrifuge. The mitochondria were subsequently pelleted from the second low speed supernatant by high speed centrifugation at 13 800 x g for 15 minutes at 4°C, using a Beckman L5-50 ultracentrifuge. Following the removal of the supernatant, the mitochondria were washed in 4ml of homogenizing buffer and re-homogenized with a Dounce homogenizer (loose-fitting pestle) using approximately 5 strokes. Following high speed centrifugation (13 800 x g, 15mins) and removal of the supernatant, the mitochondrial pellet was washed 3 more times in the case of the liver samples, and twice for the other tissues, to ensure relatively clean mitochondrial pellets.

2.1.2. Mitochondrial DNA isolation:

Mitochondrial DNA was harvested from the mitochondrial preparations by a modification of the alkaline extraction procedure outlined by Palva & Palva (1985). Initially each mitochondrial pellet was resuspended in 200µl of resuspension buffer (50mM

glucose, 10mM EDTA, 25mM Tris-HCl, pH8.0). The mitochondria were subsequently lyzed and any contaminating chromosomal DNA was denatured by the addition of 400µl of freshly prepared alkaline-SDS (0.2M NaOH, 1% SDS). These lysates were thoroughly mixed and incubated on ice for 5-10 minutes. To precipitate the SDS and denatured protein (ie mitochondrial debri), 300µl of 3M potassium acetate was added and the contents of each tube were mixed and subsequently placed at -70°C for 20-30 minutes. Following centrifugation at 10 000 x g for 5 minutes, using a benchtop microcentrifuge, 900µl of the clear supernatant was removed from each tube and the mtDNA was precipitated by the addition of 0.6 volume of cold isopropanol at -70°C for approximately 2 hours. The mtDNA pellets obtained after centrifugation at 10 000 x g for 10 minutes were then washed with 70% ethanol, air dried and resuspended in 500µl of TE buffer (10mM Tris-HCl, pH8.0, 1mM EDTA).

2.1.3. Purification of mitochondrial DNA:

(i) Phenol / chloroform / iso-amyl alcohol extraction:

An equal volume (500µl) of buffer saturated phenol, prepared according to Sambrook et al (1989), was added to each mtDNA sample. The contents of each tube were then thoroughly mixed for 10 minutes on a rotating wheel. Following high speed centrifugation (10 000 x g, 5mins), the top aqueous layer was removed and added to an equal volume of phenol / chloroform / iso-amyl alcohol (25:24:1). Again after mixing for 10 minutes on a rotating wheel and high speed centrifugation, the top aqueous layer was removed and added to an equal volume (500µl) of chloroform / iso-amyl alcohol (24:1). Following rotary mixing for 10 minutes, the bottom organic phase was discarded and the mtDNA was subsequently precipitated from the remaining aqueous layer by the addition of 0.1 volume saturated NaCl (6M), and 2 volumes of glass distilled ethanol at -70°C for 2 hours. The pellets obtained after high speed centrifugation (10 000 x g, 5mins) were

washed with 70% ethanol, air dried and subsequently resuspended in 100µl of TE (10mM Tris-HCl, pH8.0, 1mM EDTA) buffer.

(ii) Prep-A-Gene DNA purification matrix kit (BIORAD ®):

The procedure utilized was outlined by BIORAD ®, for the isolation and concentration of DNA from buffer/salt solutions (pages 5-6 of the supplied instruction manual). Briefly, 330µl of supplied binding buffer (50mM Tris-HCl, 1mM EDTA, 6M $NaClO_4$, pH7.5) was added to each DNA solution (100µl). Upon mixing of each tube, 10µl of prep-a-gene DNA matrix was added, which was resuspended by gentle agitation. Following incubation at room temperature for 5-10 minutes, the matrix, which now contained bound DNA, was pelleted by high-speed centrifugation at 10 000 x g for 30 seconds, and the resulting supernatant was discarded. The matrix pellet was then rinsed twice, using 50 pellet volumes of binding buffer at each rinse step. Following the disposal of the second binding buffer rinse, the prep-a-gene matrix pellet was washed using 3 consecutive 50 pellet volumes of supplied wash buffer (40mM Tris-HCl, 4mM EDTA, 0.8M NaCl, pH7.4, 1vol 95-100% ethanol). After the final third wash, the matrix was again pelleted by high speed centrifugation, and all traces of the wash buffer liquid were carefully removed. To elute the mtDNA from the prep-a-gene matrix, the matrix was resuspended in 100µl of supplied elution buffer (10mM Tris-HCl, 1mM EDTA, pH8.0), and placed in a 50°C water bath for 5 minutes. The matrix was then pelleted by high speed centrifugation, and the supernatant containing the DNA was transferred to a separate sterile eppendorf tube. To ensure all the DNA had been removed from the matrix beads, this elution procedure was repeated on the matrix pellet, and the second supernatant (100µl) was added to the first, yielding a total volume of 200µl of each mtDNA sample in elution buffer.

2.1.4. Quantitation of mitochondrial DNA:

All the mtDNA samples were quantitated by a TKO 100 mini-fluorometer (Hoefer Scientific Instruments). The procedure followed was that which was set out in the manual supplied by Hoefer Scientific Instruments (pages 1-3). DNA measurements in the TKO 100 were based on the binding of Hoechst 33258, a fluorescent dye, to the mtDNA and calf thymus DNA standards were used to calibrate the fluorometer for DNA quantitation. An aliquot (5µl) of each calf thymus DNA standard or mitochondrial DNA preparation was added to a TKO105 cuvette which contained 2mls of freshly prepared dye solution [0.1µg/ml Hoechst 33258 solution in 1x TNE buffer (0.1M NaCl, 10mM Tris-HCl, 1mM EDTA, pH7.4)]. After placing the cuvette within the fluorometer, the amount of mitochondrial DNA in each sample was calculated by comparing the reading obtained for that sample with that of the calf thymus DNA standards of known concentration. Following quantitation, each mtDNA sample was stored at -20°C until analyzed by the polymerase chain reaction (PCR).

2.2. Polymerase Chain Reaction (PCR) analysis:-

2.2.1. PCR reaction reagents:

All PCR experiments were performed in 0.5ml thin walled reaction tubes. Following optimization, each PCR amplification was performed on 5ng of mitochondrial DNA preparation in a final reaction volume of 50µl which contained; 2.5mM $MgCl_2$, 200µM of each deoxyribonucleoside triphosphate (dNTP), 1µM of each primer, 2.5 units of amplitaq® DNA polymerase, in a 1x PCR reaction buffer (50mM KCl, 10mM Tris-HCl, pH8.3). Master mixtures of these reagents were prepared where possible, and each set of reactions was accompanied with a negative control in which the DNA template was omitted.

Prior to commencing the PCR reaction, one drop of mineral oil was overlayed on top of the reaction mixture to prevent evaporation/condensation which might result in uneven mixing of the contents of each tube.

2.2.2. Mouse mitochondrial DNA PCR primers:

All the primers used within the PCR experiments were chemically synthesized by Bresatec Ltd., Australia, from our specified sequences. They were prepared on an Applied Biosystems 380B DNA synthesizer. Each oligonucleotide was detritylated and cleaved from the support on the synthesizer, and then desalted. Both L strand and H strand primers were synthesized and numbered according to the mouse mitochondrial DNA genome sequence derived by Bibb et al (1981). (Sequences are shown in the results section, Chapter 3)

2.2.3. PCR thermal cycling conditions:

All PCR reactions were carried out for a total of 40 cycles using a FTS-1 DNA Thermal Sequencer (Corbett Research). Unless stated otherwise, the cycle times were as follows; denaturation, 10 seconds at 92°C ; annealing, 40 seconds at 54°C ; and primer extension, 60 seconds at 72°C. In the initial cycle the denaturation step was carried out for 300 seconds, and in the final cycle, the extension time was programmed for 150 seconds.

2.2.4. Hot start PCR method:

The procedure employed for the hot start PCR reaction was a modification of that designed by Perkin Elmer / Applied Biosystems Pty. Ltd., Australia. Initially both the L and H strand oligonucleotide primers were added to their respective reaction tubes.

Following centrifugation (20 seconds, 3000 x g), a wax bead of paraplast ® tissue embedding medium was added to each tube and centrifuged to the bottom. The wax beads were then melted by placing each tube at 70°C for approximately 15 seconds. These tubes were then placed at room temperature where the wax solidified, forming a layer over the primer mixture. The following reagents were then added; PCR reaction buffer, $MgCl_2$, dNTP's, amplitaq® DNA polymerase, mtDNA template, and autoclaved H_2O (purified in a Milli Q filter system) to make a final reaction volume of 50µl. Again, one drop of mineral oil was layered on the surface and then each tube was placed within the DNA thermal cycling machine.

2.2.5. Agarose gel electrophoresis:

PCR amplified fragments were separated by agarose gel electrophoresis. From the initial 50µl PCR reaction solution, an 18µl aliquot was mixed with 2µl of gel loading buffer (0.25% bromophenol blue, 40% sucrose, 4°C), and loaded onto a 1% agarose gel made with 0.5x TBE buffer (0.045M Tris borate, 0.001M EDTA), which contained 0.5µg/ml ethidium bromide. The GNA 200 gel tank (Pharmacia Pty. Ltd.) which contained 0.5x TBE buffer was then connected with electrical leads to a GPS 200/400 power supply (Pharmacia Pty. Ltd.), and a voltage of 130 volts was applied for approximately 2 hours. The DNA bands were visualized by exposing the ethidium bromide stained gel to ultraviolet light (LKB 2011 Macrovue Transilluminator). The fragment size of the bands in each lane were estimated by comparison with a set of DNA size markers. The DNA size markers used were the EcoR1 enzyme restricted fragments of Spp-1 Bacteriophage DNA which generated 15 fragments of the following sizes; 8.51Kb, 7.35Kb, 6.11Kb, 4.84Kb, 3.59Kb, 2.81Kb, 1.95Kb, 1.86Kb, 1.51Kb, 1.39Kb, 1.16Kb, 0.98Kb, 0.72Kb, 0.48Kb and 0.36Kb respectively. (see appendix)

2.3. Genetic engineering of mouse mtDNA deletions:-

2.3.1. Computer analysis:

The complete restriction enzyme map of the mouse mtDNA genome was obtained from the data base Genebank. From this map, a list was constructed of those restriction enzymes which cut the mouse mitochondrial genome only twice.

2.3.2. Nhe1 restriction enzyme endonuclease digestion:

Mouse kidney mtDNA samples were digested with the restriction endonuclease Nhe1 (*Neisseria mucosa*). MtDNA(10µg), dissolved in 34µl of autoclaved H_2O that was purified in a Milli Q filter system, was mixed with 4µl of 10x restriction enzyme buffer (100mM Tris-HCl, 100mM $MgCl_2$, 500mM NaCl, 10mM Dithioerythritol (DTE), pH7.5, at 37°C). 2µl of the restriction endonuclease Nhe1 (10units/µl) was then added and after placing one drop of mineral oil on the surface, the tube was incubated at 37°C for 6 hours. Following incubation, the mitochondrial DNA solution was then purified (see 2.1.3(ii)).

2.3.3. T4 DNA ligase ligation reaction:

Mitochondrial DNA (8µg dissolved in 75µl of TE buffer), which had previously been digested with the restriction endonuclease Nhe1, was mixed with 8.6µl of 10x ligation buffer (Tris-Hcl 660mM, $MgCl_2$ 50mM, Dithioerythritol 10mM, ATP 10mM, pH7.5), and 2.5µl of T4 DNA ligase (1unit/µl). Following the addition of 1 drop of mineral oil, the reaction tube was placed at 16°C overnight. The mitochondrial DNA solution was then purified (see 2.1.3 (ii)), quantitated (see 2.1.4), and then subsequently analyzed by PCR (see 2.2).

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

Direct repeat sequences within the mouse mitochondrial genome of 10 basepairs or greater were located by The Australian National Genomic Information Service (ANGIS, located at The University of Sydney, NSW, 2006, Australia). The output was generated using NIP software, (Rodger Staden, MRC, Cambridge), (Gleeson et al 1991).

2.5. Buffers and reagents:-

1. Homogenizing buffer: (0.34M Sucrose, 1mM EDTA, 10mM Tris-HCl, pH7.5)

To 400mls of MilliQ H_2O (autoclaved), the following reagents were added,

Tris-HCl	0.788g
Sucrose	58.191g
EDTA	0.186g

The pH of the combined mixture was adjusted to pH7.5, and the volume was adjusted to 500mls with Milli Q H_2O (autoclaved). The final solution was then filtered through a 0.2µm millipore filter into a pre-autoclaved bottle, and stored at 4°C.

2. Resuspension buffer: (50mM glucose, 10mM EDTA, 25mM Tris-HCl, pH8.0)

To 80mls of Milli Q water (autoclaved), the following reagents were added,

Tris-HCl	0.394g
EDTA	0.372g
Glucose	0.901g

The pH of the combined mixture was adjusted to pH8.0 with concentrated NaOH, and the volume was adjusted to 100mls with Milli Q water (autoclaved). The final solution was then filtered through a 0.2µm millipore filter into a pre-autoclaved bottle, and stored at 4°C.

3. Alkaline-SDS: (0.2M NaOH, 1% SDS)

To 20mls of Milli Q water (autoclaved), the following reagents were added,

NaOH	0.16g
SDS	0.20g

The mixture was shaken until both the reagents had completely dissolved. The final solution was then stored at room temperature for periods up to 1 week.

4. TE buffer: (10mM Tris-HCl, pH8.0, 1mM EDTA)

To 400mls of Milli Q water, 0.788g of Tris-HCl was added. The pH of the solution was then adjusted to 8.0, and the volume was subsequently adjusted to 500ml with Milli Q water. EDTA (0.186g) was then added and the final solution was autoclaved, and stored at room temperature.

5. 3M Potassium Acetate:

To 88.5mls of Milli Q water, the following reagents were added,

Potassium acetate	29.442g
Glacial acetic acid	11.5mls

The final 100ml solution was then autoclaved and stored at room temperature.

6. 6M NaCl:

20.88g of NaCl was added to 100mls of Milli Q water, and the final solution was autoclaved and stored at room temperature.

7. 10x TNE buffer: (1M NaCl, 100mM Tris-HCl, 10mM EDTA, pH7.4)

To 400mls of autoclaved Milli Q water, the following reagents were added,

NaCl	29.200g
Tris-base	6.050g
EDTA	1.861g

The pH of the final combined solution was adjusted to 7.4 with concentrated HCl, and the volume was adjusted to 500mls with autoclaved Milli Q water. The solution was then filtered through a 0.2µm millipore filter into a pre-autoclaved bottle, and stored at 4°C.

8. Gel loading buffer: (0.25% bromophenol blue, 40% sucrose)

To 100mls of autoclaved Milli Q water, the following reagents were added,

Bromophenol blue	0.25g
Sucrose	40.00g

The final solution was stored at 4°C.

9. 5x TBE buffer: (0.45M Tris borate, 0.01M EDTA)

To 490mls of autoclaved Milli Q water, the following reagents were added,

Tris-base	27.00g
Boric acid	13.75g
0.5M EDTA, pH8.0	10mls

The final solution was stored at room temperature.

2.6. Chemicals:-

1. Agarose
   Sigma Chemical Company, St. Louis, Missouri, USA

2. Amplitaq® DNA polymerase
   Perkin Elmer / Applied Biosystems Pty. Ltd., Australia

3. Boric acid

   BDH Chemicals Ltd., Poole, England

4. Bromophenol blue

   Sigma Chemical Company, St. Louis, Missouri, USA

5. Calf thymus DNA (high molecular weight)

   Boehringer Mannheim Australia Pty. Ltd., Castle Hill, NSW, Australia

6. Chloroform

   Ajax Chemicals, Auburn, New South Wales, Australia

7. Deoxyribonucleoside triphosphate's (dNTP's)

   Perkin Elmer / Applied Biosystems Pty. Ltd., Australia

8. EcoR1 restricted spp-1 Bacteriophage DNA size markers

   Bresatec Ltd., Adelaide, South Australia, Australia

9. EDTA (ethylenediaminetetraacetic acid, disodium, dihydrate)

   Sigma Chemical Company, St. Louis, Missouri, USA

10. Ethanol

    Ajax Chemicals, Auburn, New South Wales, Australia

11. Ether (Anaesthetic ether)

    Ajax Chemicals, Auburn, New South Wales, Australia

12. Ethidium bromide

Sigma Chemical Company, St. Louis, Missouri, USA

13. Glucose (D-glucose)

Ajax Chemicals, Auburn, New South Wales, Australia

14. Hoechst 33258 dye

Hoefer Scientific Instruments, San Francisco, California, USA

15. Iso-amyl alcohol

Ajax Chemicals, Auburn, New South Wales, Australia

16. Isopropanol (propan-2-ol)

Ajax Chemicals, Auburn, New South Wales, Australia

17. Liquid nitrogen

Commonwealth Industrial Gases Ltd., Torrensville, South Australia, Australia

18. Magnesium chloride ($MgCl_2$), 25mM

Perkin Elmer / Applied Biosystems Pty. Ltd., Australia

19. Mice (*Balb C*)

10 week / 19 month old
IMVS Animal Resource Centre, Gilles Plains, South Australia, Australia

42 month old
A gift from Prof. Alexander A. Morley, Dept. of Haematology, Flinders Medical Centre, South Australia, Australia

20. Mineral oil

Perkin Elmer / Applied Biosystems Pty. Ltd., Australia

21. Nhe1 restriction endonuclease (10 units/µl)

Boehringer Mannheim Australia Pty. Ltd., Castle Hill, NSW, Australia

22. Nhe1 restriction endonuclease buffer (10x)

Boehringer Mannheim Australia Pty. Ltd., Castle Hill, NSW, Australia

23. Oligonucleotide Primers

Bresatec Ltd., Adelaide, South Australia, Australia

24. Paraplast® tissue embedding medium (wax)

Oxford® Labware, Division of Sherwood Medical, St. Louis, Missouri, USA

25. PCR reaction buffer (10x)

Perkin Elmer / Applied Biosystems Pty. Ltd., Australia

26. Phenol

Sigma Chemical Company, St. Louis, Missouri, USA

27. Potassium acetate

BDH Chemicals Ltd., Poole, England

28. Prep-A-Gene® DNA purification kit

BIO-RAD Laboratories, Hercules, California, USA

29. SDS (sodium dodecyl sulfate)-sodium salt
Sigma Chemical Company, St. Louis, Missouri, USA

30. Sodium chloride
Ajax Chemicals, Auburn, New South Wales, Australia

31. Sodium hydroxide
Ajax Chemicals, Auburn, New South Wales, Australia

32. Sucrose
Sigma Chemical Company, St. Louis, Missouri, USA

33. T4 DNA ligase (1unit/µl)
Boehringer Mannheim Australia Pty. Ltd., Castle Hill, NSW, Australia

34. T4 DNA ligase buffer (10x)
Boehringer Mannheim Australia Pty. Ltd., Castle Hill, NSW, Australia

35. Trizma® base (Tris[hydroxymethyl] aminomethane)
Sigma Chemical Company, St. Louis, Missouri, USA

36. Trizma® hydrochloride (Tris[hydroxymethyl] aminomethane hydrochloride)
Sigma Chemical Company, St. Louis, Missouri, USA

Chapter 3:

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

3.0. Introduction:-

Within this chapter, a Polymerase Chain Reaction (PCR) method, specific for the mouse mitochondrial genome, was developed to subsequently determine if deleted mtDNA molecules accumulate within the ageing mouse. The characteristics of the polymerase chain reaction are such that the reaction conditions vary for each different application. These conditions are dependent upon a number of factors which include, the size and base composition of the primers and the DNA template, the size of the required PCR product, and the type of DNA polymerase used to synthesize the DNA, to mention only a few. Thus, each new application of the PCR technique requires optimization of each of the reaction conditions to ensure the maximum synthesis of the desired PCR product, whilst limiting the production of non-specific background bands. These variable conditions which need to be optimized include the following; the magnesium (Mg^{2+}) concentration, the Taq DNA polymerase concentration, the amount of template DNA, the concentration of each primer, the concentration of each dNTP, the PCR denaturation, annealing and extension times, and the PCR denaturation, annealing and extension temperatures.

In developing this PCR method, PCR primers specific for both strands of the mouse mitochondrial genome were designed so that the major region between the two origins of replication could be scanned for the presence of deletions. Following the synthesis of these designed mouse PCR primers, the previously mentioned PCR

reaction conditions were optimized so that detectable PCR products could be visualized by agarose gel electrophoresis.

Due to the limited knowledge of the size, position and number of deletions which might exist within the mouse mitochondrial genome of aged mice, a PCR scheme was developed to methodically screen the entire major region of the genome between the two origins of replication using multiple primer pair combinations. This two-part PCR method was based upon the principles of the primer shift method devised by Sato et al (1989), to confirm if the bands generated by each primer pair were genuine PCR products. In order to validate that this PCR method was capable of detecting deleted mitochondrial DNA molecules, this scheme was used to examine genetically engineered heteroplasmic samples which contained both wildtype and deleted mouse mtDNA genomes.

3.1. Design of mouse mitochondrial DNA primers:-

Experimental design:-

Mouse mtDNA primers were designed by analyzing the sequences and location of PCR primers which have previously been used for detecting deletions within the human genome, and comparing them to the corresponding regions within the mouse mitochondrial genome. Those sequences which showed a high degree of homology between the two species (≥ 80%), were considered as possible sites for mouse PCR primers. These mouse mtDNA sequences were then further analyzed for: %GC content, to avoid sequences with runs (3 or more) of C's or G's at the 3' end; hairpin structures; non-tRNA regions; palindromic sequences; inverted direct repeats, and their T_ms (denaturation temperature) values were calculated by the rule-of-thumb calculation

of 2°C for A or T and 4°C for G or C (Thein et al 1986). The number of nucleotides in each primer was then adjusted so that the T_ms values of all the primers were similar (within 2°C). All possible primer pairs were then analyzed for 3' complementary regions. Overall, both L (light strand) and H (heavy strand) primers were designed, and numbered according to the mouse mitochondrial genome sequence derived by Bibb et al (1981).

Results:-

The sequences and nucleotide positions of the designed mouse mitochondrial DNA PCR primers are listed in Table 3.1.1. Each primer was numbered according to the mouse mtDNA sequence derived by Bibb et al (1981), and the first letter of each primer (L or H), specifies whether the sequence is located on the light or the heavy strand of the mtDNA genome. In total 10 primers were designed, 4 carrying light strand sequences, and 6 comprising of heavy strand sequences.

The characteristics of each primer are summarized in Table 3.1.2. All the primers were chosen within protein coding genes except for the primer H6 which was located within both the 12sRNA gene and the $tRNA^{phe}$ gene. Each primer was between 19 to 22 nucleotides in length, and showed at least 80% homology with the corresponding region in the human mitochondrial genome sequence derived by Anderson et al (1981), except for the primer L480 which shared 72.72% homology. The %GC content of the primers ranged from 28.6% to 57.9%. From analyzing the sequences of these primers, they appeared to contain no hairpin structures, inverted direct repeats or palindromic sequences. Their calculated T_ms values were either 56°C or 58°C, except for the primer H1452 which had a T_ms value of 60°C. All possible L and H primer pair combinations were analyzed and were found to contain no 3' complementary sequences.

Table 3.1.1. Designed mouse mtDNA PCR primers:
This table shows the sequences and nucleotide positions of 10 designed mouse mtDNA oligonucleotide PCR primers. The first letter of each primer, L or H, specifies its priming strand *, and the numbering of each mtDNA nucleotide is according to the sequence derived by Bibb et al (1981) **. Each primer is listed in the 5'→ 3' direction.

Primers *	Sequence 5'→ 3'	Nucleotide position in mtDNA**
L480	TTATTCCACTTCACTAACAATA	4804 to 4826
L567	AATAGTAGAAGCAGGAGCAG	5675 to 5694
L821	TACCCAACTATCCATAAATCT	8211 to 8231
L1107	AACTCCATGAAGCTTCATAG	11072 to 11091
H6	CCAAACCTTTGTGTTTATGG	79 to 60
H732	GTCAGTATATTCGTAGCTTCA	7348 to 7328
H821	AGATTTATGGATAGTTGGGTA	8231 to 8211
H1107	CTATGAAGCTTCATGGAGTT	11091 to 11072
H1295	AGTGTAATTAGTAGGGCTCA	12973 to 12954
H1452	CCTCATGGAAGGACGTAGC	14545 to 14527

Table 3.1.2. Characteristics of the designed mouse mtDNA primers:
This table summarizes the characteristics of each mouse mtDNA PCR primer. The position of each primer is listed in accordance with the sequence derived by Bibb et al (1981), and the corresponding gene location is sited. [ND2, ND4 and ND5 are subunits 2, 4 and 5 of NADH dehydrogenase (Complex I), COXI and COXII are subunits 1 & 2 of Cytochrome c oxidase (Complex IV), ATPase 6 is subunit 6 of ATPase Synthase (Complex V), 12srRNA is 12s ribosomal RNA, $tRNA^{phe}$ is transfer RNA^{phe}, and cyt.b is cytochrome b, a subunit of Ubiquinol-Cytochrome c oxidoreductase (Complex III)]. The size of each primer is expressed as the number of nucleotides (nts) in each sequence, and the number of C+G residues in each primer sequence is expressed as a % of the total number of residues. The % homology each primer shares with the human mtDNA sequence is also listed. The T_ms (denaturation temperature) value of each primer was also calculated by the rule-of-thumb method (Thein et al 1986).

Primer:	Location:	Position:	Size:	%homology:	T_ms:	% G+C:
L480	ND2	4804 - 4826	22nts	72.72%	56°C	28.6%
L567	COX I	5675 - 5694	20nts	80%	58°C	45%
L1107	ND4	11072-11091	20nts	80%	56°C	35%
L821	ATPase 6	8211 - 8231	21nts	85.71%	56°C	33.3%
H6	12srRNA-$tRNA^{Phe}$	79 - 60	20nts	90%	56°C	40%
H732	COX II	7348 - 7328	21nts	80.95%	58°C	38.1%
H821	ATPase 6	8231 - 8211	21nts	85.71%	56°C	33.3%
H1107	ND4	11091-11072	20nts	80%	56°C	35%
H1295	ND5	12973-12954	20nts	80%	56°C	40%
H1452	Cyt. b	14545-14527	19nts	84.21%	60°C	57.9%

Summary:-

Following the analysis of PCR primers which have previously been used for amplifying regions of the human mitochondrial genome, both H (heavy) and L (light) strand primers, of 18-22 nucleotides in length, located within protein coding genes (except H6), were designed for the mouse mitochondrial genome. The relative position of each of these primers can be more clearly visualized by looking at the Diagram 3.1, which shows the genetic map of the mouse mitochondrial genome and displays the gene locations and relative positioning of the primers which span the major region between the two origins of replication.

The following table summarizes the different primer pair combinations used within this thesis and the expected/predicted PCR fragment sizes which would be generated by these primers annealing to intact (non-deleted) mouse mitochondrial DNA. The first letter of each primer, L or H, specifies its priming strand, and the numbering of each mtDNA nucleotide is according to the mouse mitochondrial DNA sequence derived by Bibb et al (1981).

Primer pair combination: L strand		H strand	Expected PCR product: (bp)
L480	-	H732	2544
"	-	H821	3427
"	-	H1107	6287
"	-	H1295	8169
"	-	H1452	9741
"	-	H6	11570
L567	-	H732	1673
"	-	H821	2556
"	-	H1107	5416
"	-	H1295	7298
"	-	H1452	8870
"	-	H6	10699

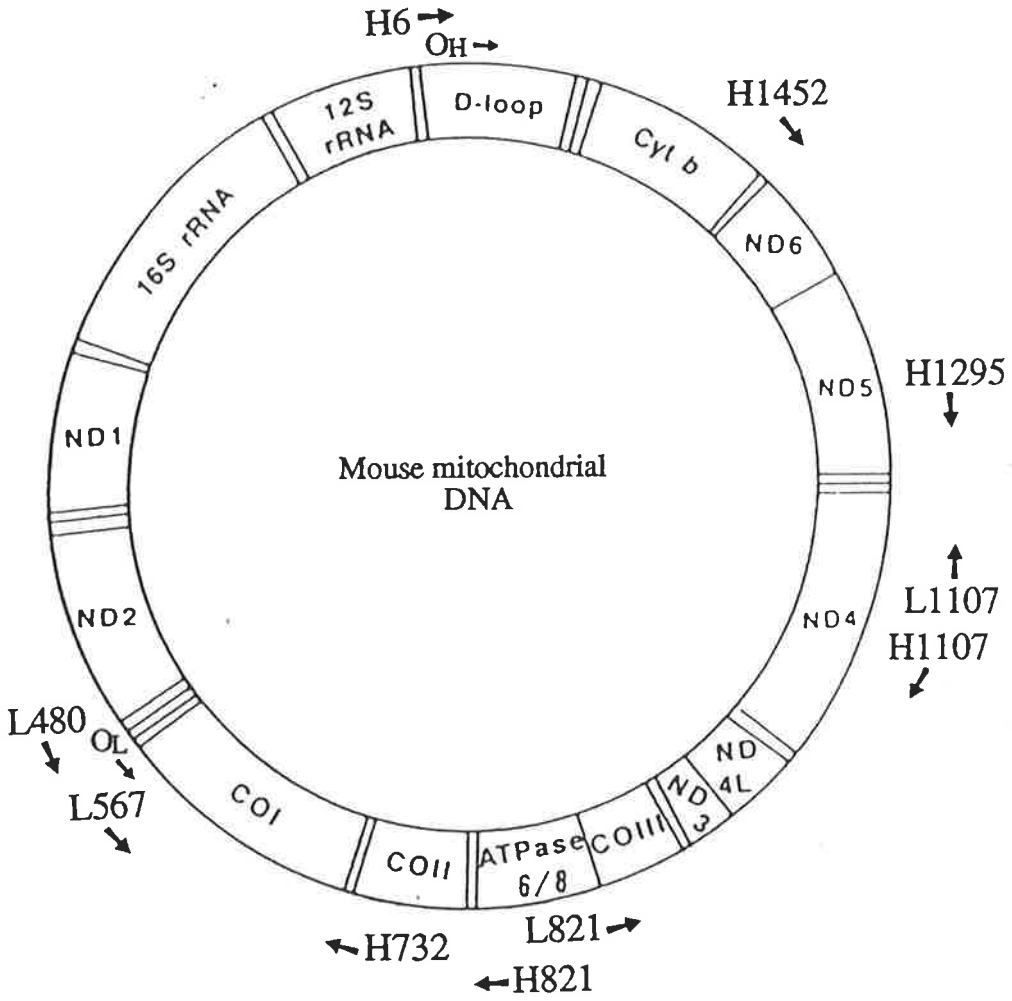


Diagram 3.1. The genetic map of the mouse mitochondrial genome:
This diagram represents the genetic map of the mouse mitochondrial genome, showing the relative spatial location and orientation (5'→ 3') of each L and H strand PCR primer. The first letter, L or H, specifies the priming strand, and the numbering is according to the sequence derived by Bibb et al (1981). The open bars represent tRNA (transfer RNA) genes. 12srRNA and 16sRNA are ribosomal RNA genes. ND1, ND2, ND3, ND4L, ND4, ND5 and ND6 genes code for subunits 1, 2, 3, 4L, 4, 5 and 6 of NADH dehydrogenase (Complex I), COI, COII and COIII genes code for subunits 1, 2 and 3 of Cytochrome c oxidase (Complex IV), the Cyt. b gene codes for Cytochrome b, a subunit of Ubiquinol-cytochrome c oxidoreductase (Complex III), and ATPase 6 and 8 genes code for 2 subunits of ATPase Synthase (Complex V). The light and heavy strand origins of replication are denoted as O_L and O_H respectively.

3.2. Optimization of the Polymerase Chain Reaction conditions:-

Experimental design:-

Due to the limited knowledge of the size and exact location of any deletions within the mouse mitochondrial genome, it was difficult to predict the size of the PCR products that would need to be synthesized. Therefore, the PCR conditions were optimized for the largest PCR product which could be successfully amplified within our system. This involved the primers L821 and H1295 which were capable of amplifying a 4.762Kb PCR product. Following the optimization of the various PCR parameters using this primer pair on 10 week old mouse liver mtDNA, the remaining mouse PCR primers were then analyzed to confirm that they were also capable of synthesizing PCR products under these optimized conditions.

Standard PCR conditions from human PCR studies were originally employed which included; 2.5mM $MgCl_2$, 200µM of each dNTP, 1µM of each primer, 2 units of Taq DNA polymerase, and 2ng of mtDNA preparation in a total reaction volume of 50µl. The initial thermal cycling conditions were as follows; a denaturation phase of 94°C for 60 seconds, an annealing phase of 52°C for 60 seconds, and an extension phase of 72°C for 180 seconds. These reactions were carried out for a total of 40 cycles, and then an aliquot was subsequently removed and loaded onto an ethidium bromide stained agarose gel.

Each of the above PCR parameters were individually optimized to generate the maximum yield of the 4.762Kb PCR product. Following the optimization of any given parameter, the most favourable concentration, time or temperature obtained was then used in the optimization of subsequent parameters. After all the conditions had been adjusted, the remaining PCR primers were analyzed by using different primer pair combinations and the products obtained were confirmed by size and primer shift analysis.

Results:-

3.2.1. Optimization of the Taq DNA polymerase and $MgCl_2$ concentrations:

Using standard PCR conditions, the effect of changing the amount of DNA polymerase and the $MgCl_2$ concentration, on the synthesis of the L821-H1295 PCR product was examined. The results are shown in Figure 3.2.1. With 1.5 units of DNA polymerase in the reaction mixture (lanes 2-5), the expected 4.7Kb PCR product could not be significantly amplified until the $MgCl_2$ concentration was increased to 3mM (lane 5). Even at this concentration of $MgCl_2$, the PCR product was only represented by a faint band within the gel. Increasing the amount of DNA polymerase to 2 units (lanes 6-9), enabled the PCR product to be detected at a lower $MgCl_2$ concentration of 2.5mM (lane 8). The intensity of this 4.7Kb band slightly increased when the $MgCl_2$ concentration was increased to 3mM (lane 9), however this band was still relatively faint. Further increasing the amount of enzyme to 2.5 units (lanes 10-13), enabled the 4.7Kb PCR product to be amplified at an even lower $MgCl_2$ concentration of 2mM (lane 11). The intensity of this 4.7Kb band significantly increased with the addition of 2.5mM $MgCl_2$ (lane 12), however further increasing the $MgCl_2$ concentration to 3mM (lane 13) resulted in a slight reduction in the 4.7Kb band intensity with the co-production of several non-specific PCR products.

3.2.2. Optimization of the mitochondrial DNA template concentration:

Using the optimal concentrations of Taq DNA polymerase enzyme and $MgCl_2$, the amount of initial mtDNA template added to the reaction mixture was altered (Figure 3.2.2). As expected, no PCR product was detected when no DNA template was added to the reaction (lane 2). Adding 0.1ng of mtDNA template to the reaction resulted in a faint streaking pattern being observed with no 4.7Kb product (lane 3). However, this PCR product was amplified by further increasing the amount of template to 1 and 2ng (lanes 4 and 5 respectively), with the maximum amount of product being synthesized

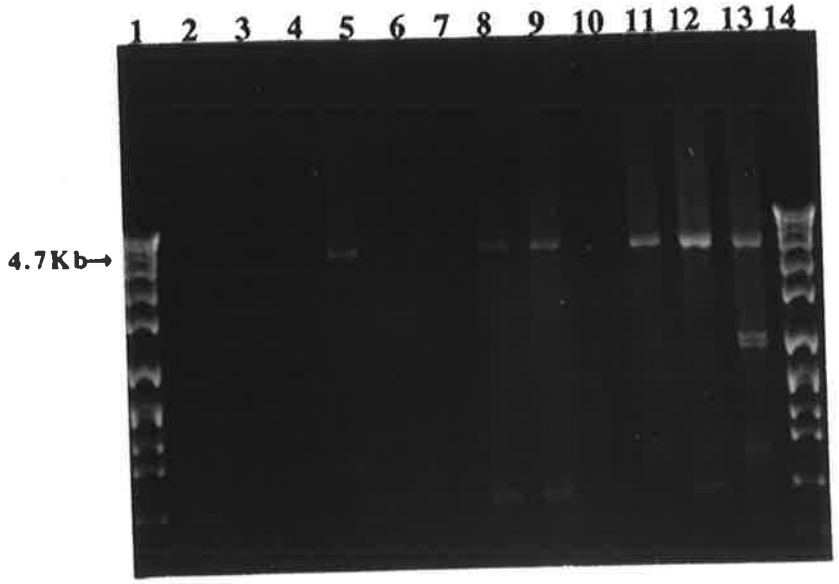


Figure 3.2.1. Optimization of the Taq DNA polymerase and $MgCl_2$ concentrations: Using standard PCR conditions, 10 week old mouse liver mtDNA was amplified with the primers L821 and H1295. Lanes 2-5 represent PCR experiments where 1.5 units of Taq DNA polymerase was added in combination with 1.5mM, 2.0mM, 2.5mM and 3mM $MgCl_2$ respectively. Lanes 6-9, show the results obtained when 2 units of Taq DNA polymerase was added in combination with 1.5mM, 2.0mM, 2.5mM and 3mM $MgCl_2$ respectively. Finally, lanes 10-13 represent PCR experiments where 2.5 units of Taq DNA polymerase was added in combination with 1.5mM, 2.0mM, 2.5mM and 3mM $MgCl_2$ respectively. Lanes 1 & 14 represent DNA size markers which range in size from 8.5Kb - 0.36Kb.

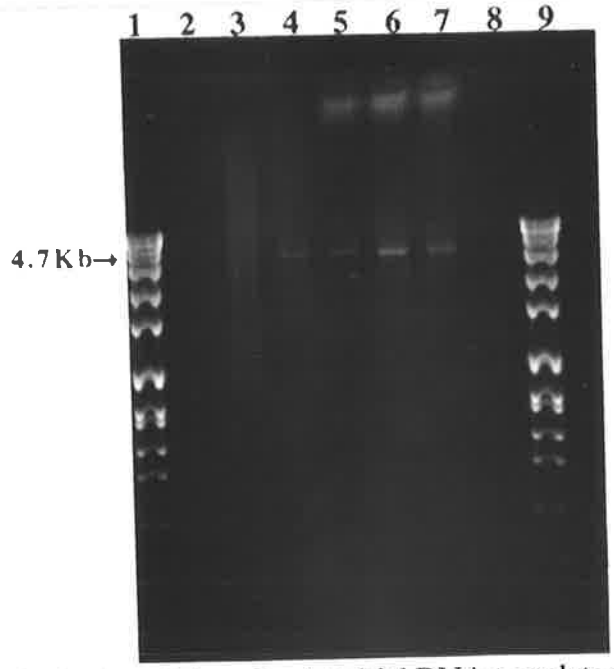


Figure 3.2.2. Optimization of the mitochondrial DNA template concentration: Using standard PCR conditions in combination with the previously optimized parameters, various amounts of 10 week old mouse liver mtDNA template of 0, 0.1, 1, 2, 5, 10 and 100ng (lanes 2-8 respectively), were added to PCR reactions containing the primers L821 and H1295. Lanes 1 & 9 represent DNA size markers which range in size from 8.5Kb - 0.36Kb.

with the addition of 5ng of mtDNA preparation (lane 6). Further increasing the amount of DNA template resulted in a slight reduction in yield when 10ng was added (lane 7), while no product was observed with a further increment in the amount of mtDNA template to 100ng (lane 8).

3.2.3. Optimization of the concentration of each primer:

Using the previously optimized conditions, the concentration of each primer required to generate the maximum yield of the L821-H1295 PCR product was determined. The results can be observed in Figure 3.2.3. With the addition of 0.25μM of each primer to the initial reaction mixture (lane 2), only a very faint band corresponding to the expected PCR product could be detected against a background streaking pattern. Doubling the concentration of each primer to 0.5μM enabled the 4.7Kb product to be more clearly visualized (lane 3). The synthesis of this PCR product was further enhanced by again doubling the concentration of each primer to 1μM (lane 4), however no subsequent increase in band intensity was observed when 2μM of each primer was added to the reaction mixture (lane 5).

3.2.4. Optimization of the concentration of each dNTP:

Using the previously optimized PCR conditions, the most favourable concentration of each dNTP was determined with respect to generating the maximum amount of the L821-H1295 PCR product (Figure 3.2.4). With an initial concentration of 50μM of each dNTP (lane 2), only a very faint streaking pattern was observed. Doubling the concentration of each dNTP to 100μM (lane 3) resulted in a clearly visible 4.7Kb PCR product being obtained. The intensity of this 4.7Kb band was further enhanced when the concentration of each dNTP was increased to 200μM (lane 4), however, further doubling the dNTP concentrations to 400μM resulted in no 4.7Kb PCR product being amplified (lane 5).

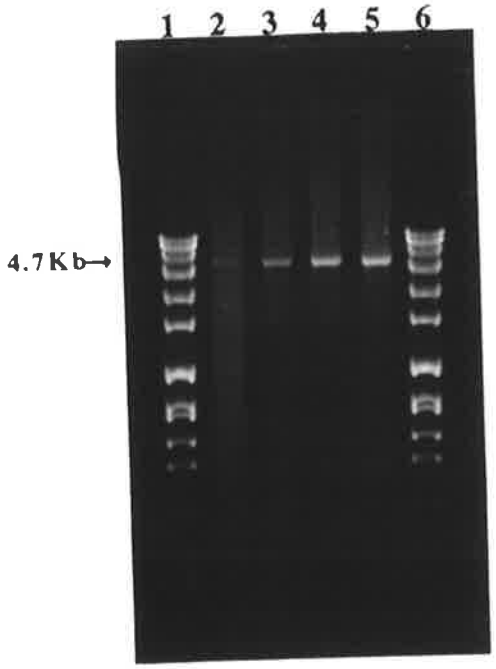


Figure 3.2.3. Optimization of the concentration of each primer:
Using standard PCR conditions in combination with the previously optimized parameters, various concentrations of each PCR primer of 0.25, 0.5, 1.0 and 2.0μM (lanes 2-5 respectively), were added to PCR reactions where 10 week old mouse liver mtDNA was amplified by the primers L821 and H1295. Lanes 1 & 6 represent DNA size markers which range from 8.5Kb - 0.36Kb.

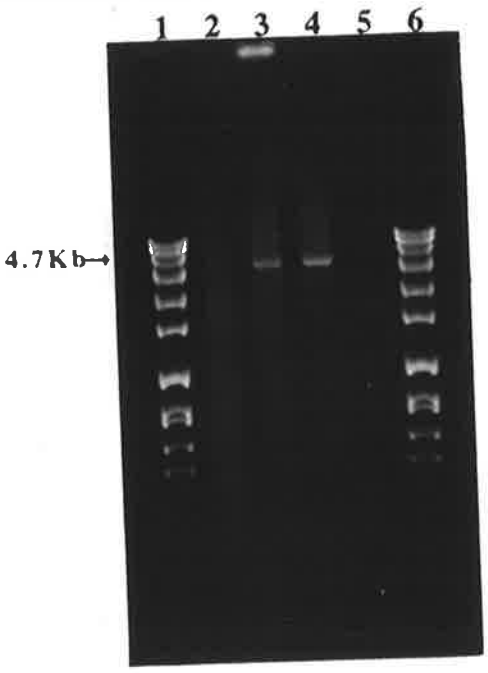


Figure 3.2.4. Optimization of the concentration of each dNTP:
Using standard PCR conditions in combination with the previously optimized parameters, various concentrations of each dNTP of 50, 100, 200 and 400μM (lanes 2-5 respectively), were added to PCR reactions where 10 week old mouse liver mtDNA was amplified by the primers L821 and H1452. Lanes 1 & 6 represent DNA size markers which range from 8.5Kb - 0.36Kb.

3.2.5. Optimization of the PCR extension time:

Using the previously optimized PCR conditions, various PCR extension times were analyzed to determine the time required to generate the maximum yield of the 4.7Kb PCR product (Figure 3.2.5). Extension times of up to 40 seconds (lanes 2-4), were found to generate negligible amounts of the PCR product. Increasing the time to 60 seconds (lane 5) enabled the product to be more clearly visualized, however the intensity of this 4.7Kb band did not increase by further increasing the PCR extension time to 80 seconds (lane 6).

3.2.6. Optimization of the PCR denaturation time:

Using the previously optimized PCR conditions, various PCR denaturation times were performed to determine the time required to enable maximum synthesis of the L821-H1295 PCR product (Figure 3.2.6). Decreasing the denaturation time from 80 seconds to 10 seconds (lanes 6-2 respectively), greatly enhanced the intensity of the 4.7Kb band, with a maximum yield being obtained with a denaturation time of 10 seconds (lane 2).

3.2.7. Optimization of the PCR annealing time:

Using the previously optimized PCR conditions, various annealing times were examined to determine the time which would result in the greatest synthesis of the L821-H1295 PCR product (Figure 3.2.7). With an annealing time of 80 seconds, no PCR product was observed (lane 5). By reducing the annealing time to 60 seconds, a 4.7Kb band corresponding to the expected PCR product was detected (lane 4), which increased in intensity by further decreasing the annealing time to 40 seconds (lane 3). However, the yield of this 4.7Kb PCR product was significantly reduced by an even further reduction in the annealing time to 20 seconds (lane 2).

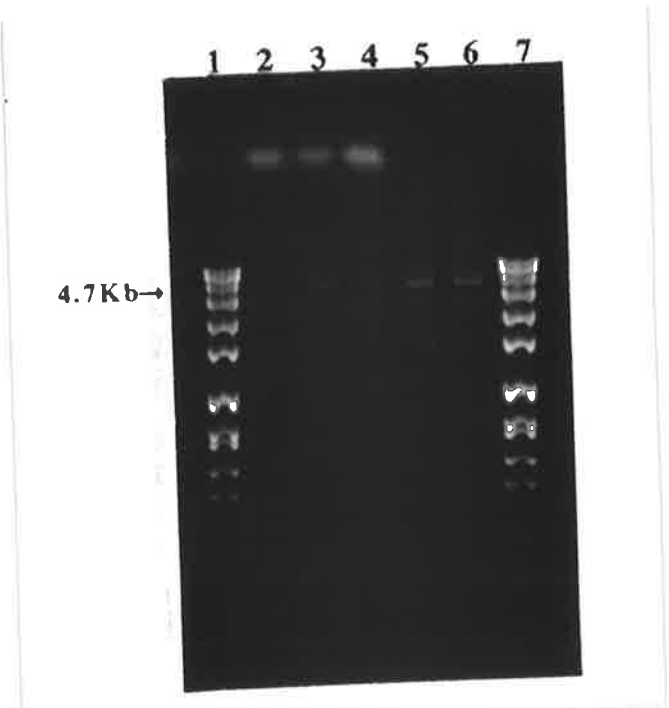


Figure 3.2.5. Optimization of the PCR extension time:
Using standard PCR conditions in combination with the previously optimized parameters, various extension times of 10, 20, 40, 60 and 80 seconds (lanes 2-6 respectively), were examined in PCR experiments where 10 week old mouse liver mtDNA was amplified by the primers L821 and H1295. Lanes 1 & 7 represent DNA size markers which range from 8.5Kb - 0.36 Kb.

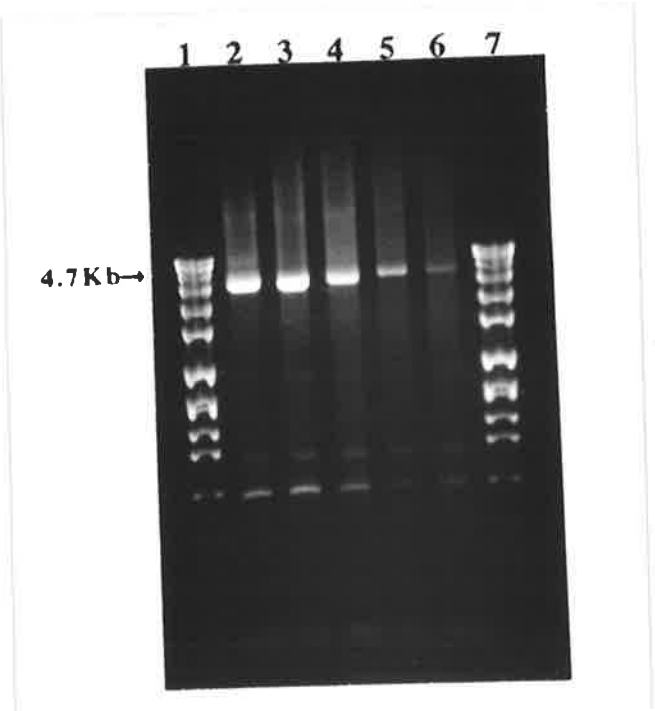


Figure 3.2.6. Optimization of the PCR denaturation time:
Using standard PCR conditions in combination with the previously optimized parameters, various denaturation times of 10, 20, 40, 60 and 80 seconds (lanes 2-6 respectively), were examined in PCR experiments where 10 week old mouse liver mtDNA was amplified by the primers L821 and H1295. Lanes 1 & 7 represent DNA size markers which range from 8.5Kb - 0.36Kb.

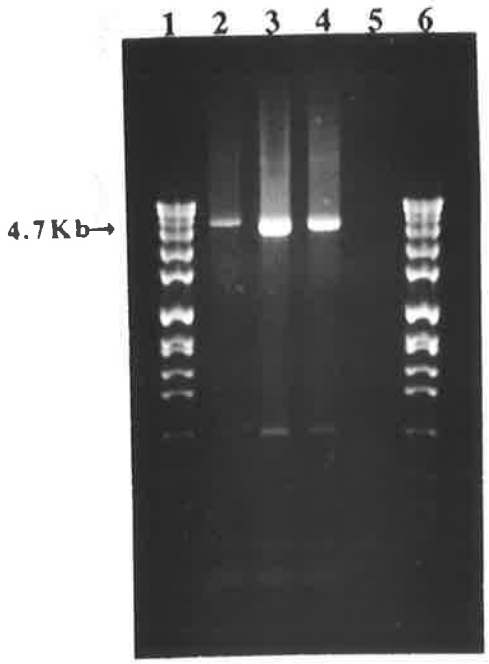


Figure 3.2.7. Optimization of the PCR annealing time:
Using standard PCR conditions in combination with the previously optimized parameters, various annealing times of 20, 40, 60 and 80 seconds (lanes 2-5 respectively), were examined in PCR experiments where 10 week old mouse liver mtDNA was amplified by the primers L821 and H1295. Lanes 1 & 6 represent DNA size markers which range from 8.5Kb - 0.36Kb.

3.2.8. Optimization of the PCR extension temperature:

Using the previously optimized conditions, the PCR extension temperature was examined (Figure 3.2.8). With an extension temperature of 76°C (lane 4), no band of 4.7Kb could be detected, implying that no PCR product had been amplified. Decreasing the extension temperature to 74°C resulted in the production of a very faint 4.7Kb band (lane 3), which significantly increased in intensity with a further reduction in the temperature to 72°C (lane 2).

3.2.9. Optimization of the PCR denaturation temperature:

Using the previously optimized conditions, the denaturation temperature was examined (Figure 3.2.9). With a denaturation temperature of 96°C, no PCR product could be observed (lane 4). However, the L821-H1295 PCR product was amplified by reducing the denaturation temperature to 94°C (lane 3). The intensity of this 4.7Kb band slightly increased with a further reduction in the temperature to 92°C (lane 2).

3.2.10. Optimization of the PCR annealing temperature:

Using the previously optimized conditions, the effect of altering the annealing temperature on the synthesis of the 4.7Kb PCR product was examined (Figure 3.2.10). By serially reducing the annealing temperature by 2°C from 58°C to 52°C (lanes 5-2 respectively), the amount of PCR product formed gradually increased. Concomitant with this increase in PCR product yield, there was also an increase in the intensity of a non-specific PCR product band of approximately 0.7Kb which can be clearly seen in lane 2.

3.2.11. Analysis of all primers using optimized PCR conditions:

Using all the optimized PCR conditions, the remaining mouse mtDNA PCR primers were analyzed for their ability to generate PCR products (Figure 3.2.11). In

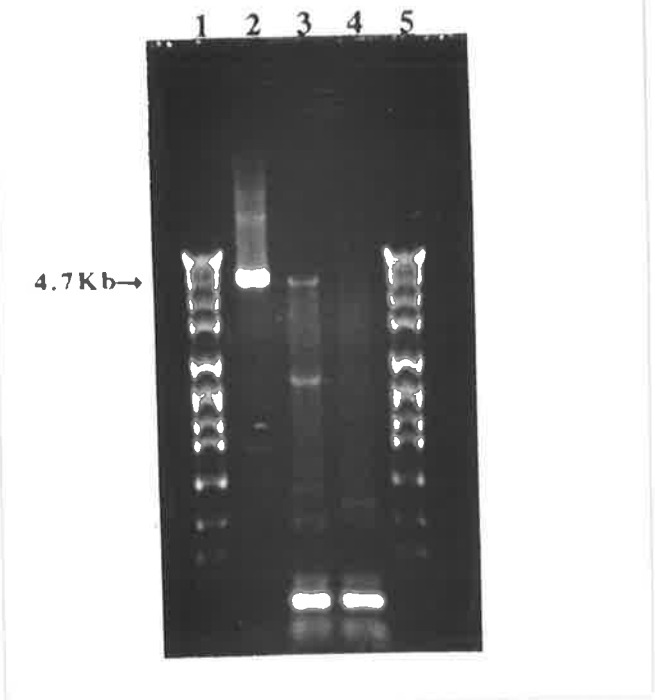


Figure 3.2.8. Optimization of the PCR extension temperature:
Using standard PCR conditions in combination with the previously optimized parameters, various extension temperatures of 72, 74 and 76°C (lanes 2-4 respectively), were examined in PCR experiments where 10 week old mouse liver mtDNA was amplified by the primers L821 and H1295. Lanes 1 & 5 represent DNA size markers which range from 8.5Kb to 0.36Kb.

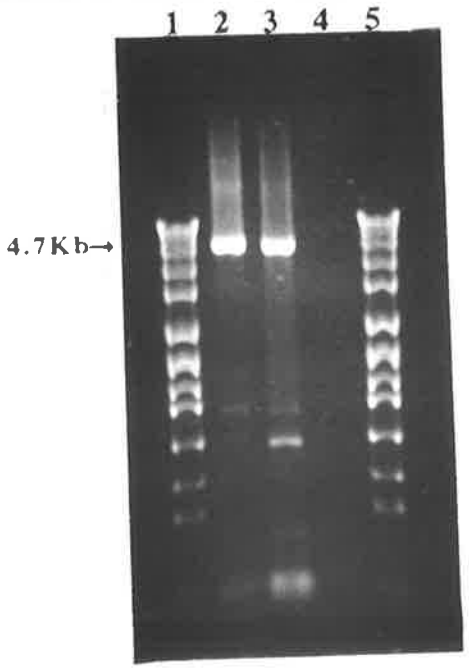


Figure 3.2.9. Optimization of the PCR denaturation temperature:
Using standard PCR conditions in combination with the previously optimized parameters, various denaturation temperatures of 92, 94 and 96°C (lanes 2-4 respectively), were examined in PCR experiments where 10 week old mouse liver mtDNA was amplified by the primers L821 and H1295. Lanes 1 & 5 represent DNA size markers which range from 8.5Kb - 0.36Kb.

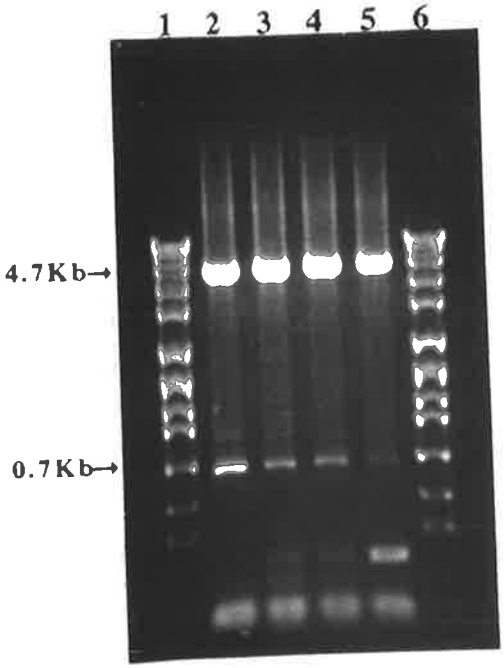


Figure 3.2.10. Optimization of the PCR annealing temperature:
Using standard PCR conditions in combination with the previously optimized parameters, various annealing temperatures of 52, 54, 56 and 58°C (lanes 2-5 respectively), were examined in PCR experiments where 10 week old mouse liver mtDNA was amplified by the primers L821 and H1295. Lanes 1 & 6 represent DNA size markers which range from 8.5Kb - 0.36Kb.

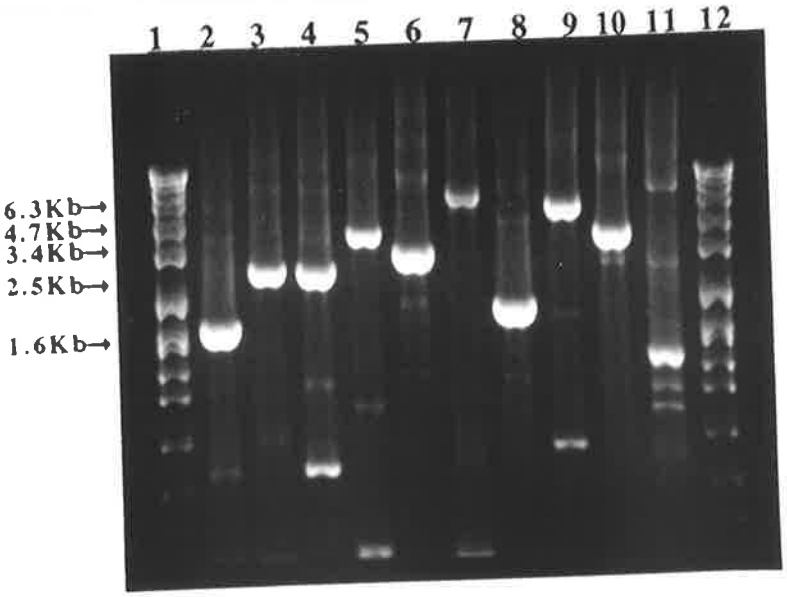


Figure 3.2.11. Analysis of Mouse mtDNA primers using optimized PCR conditions:
Using the optimized PCR conditions, 10 week old mouse liver mtDNA was amplified by various primer pairs. Lanes 2, 3 and 7, correspond to the amplification of mtDNA by the primer L567 with primers H732, H821 and H1107 respectively. Lanes 4 and 5, represent PCR reactions which contained the primer L480 in combination with the primers H732 and H821 respectively. MtDNA amplifications involving the primer L821 with the primers H1107, H1295 and H1452 are shown in lanes 6, 9 and 11 respectively. Lanes 8 and 10, represent PCR reactions which contained the primer L1107, in combination with the primers H1295 and H1452 respectively. DNA size markers ranging from 8.5Kb - 0.36Kb are displayed in lanes 1 and 12.

lanes 2, 3 and 7, DNA bands of approximately 1.6Kb, 2.5Kb and 5.4Kb respectively were detected which correspond in size to the expected PCR products which would be generated by the primer L567 with the primers H732, H821 and H1107 respectively. In lanes 6, 9 and 11, DNA bands of approximately 2.8Kb, 4.7Kb and 6.3Kb respectively were observed which correspond in size to the expected PCR products which would be amplified by the primer L821 with the primers H1107, H1295 and H1452 respectively. In lanes 4 and 5 DNA bands of 2.5Kb and 3.4Kb respectively were observed, which correspond in size to the expected PCR products derived from the interaction of the primer L480 with the primers H732 and H821 respectively. In lanes 8 and 10, DNA bands of 1.9Kb and 3.4Kb respectively were detected which correspond in size to the expected PCR products which would be synthesized by the primer L1107 with the primers H1295 and H1452 respectively.

Summary:-

Using 10 week old mouse liver mitochondrial DNA, optimal PCR conditions were characterized for the maximum amplification of the 4.7Kb product derived by the primers L821 and H1295. The final optimized conditions are summarized in Table 3.2, and were found to be as follows; 2.5mM $MgCl_2$; 2.5 units of Taq DNA polymerase; 5ng of mouse mtDNA preparation; 1μM of each primer; 200μM of each dNTP, with thermal cycling conditions of; denaturation at 92°C for 10 seconds; annealing at 54°C for 40 seconds; and an extension temperature of 72°C for 60 seconds. Under these optimal conditions, all the mouse mtDNA primers were found to be capable of generating PCR products, with the smaller PCR products showing a greater band intensity.

Table 3.2. Optimal mouse mtDNA PCR conditions:
This table summarizes the optimal PCR conditions which result in the maximum synthesis of the 4.7Kb product being amplified from 10 week old mouse liver mtDNA by the primers L821 and H1295. All the other mouse mtDNA PCR primers were also found to function under these conditions.

Taq DNA polymerase concentration	2.5 units
Magnesium ($MgCl_2$) concentration	2.5mM
Amount of mtDNA template	5ng
Concentration of each primer	1μM
Concentration of each dNTP	200μM
Extension time	60 seconds
Denaturation time	10 seconds
Annealing time	40 seconds
Extension temperature	72°C
Denaturation temperature	92°C
Annealing temperature	54°C

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

Experimental design:-

A two part PCR method for detecting deletions within the mouse mitochondrial genome was developed, based upon the principles of the primer shift PCR method (Sato et al 1989), and involving 6 of the designed mouse PCR primers. Part A, the first part of this method, is shown in Diagram 3.3.1. This is a one-way primer shift method which involves using one L strand primer, L567, in combination with 4 consecutive H strand primers, (H732, H1107, H1295 and H1452), which are dispersed within the major region between the two origins of replication. Hence, this initial part involves 4 primer pair reactions of L567-H732, L567-H1107, L567-H1295 and L567-H1452, which differ by an increasing shift in the H strand primer, and should generate PCR products of 1.673Kb, 5.416Kb, 7.298Kb and 8.870Kb respectively.

The 1.673Kb product (L567-H732) acts as a positive control for monitoring the relative amount of mtDNA within each DNA sample, providing that a deletion does not delete one or both of these primers. The larger, expected PCR products of the other 3 primer pairs may not be amplified since the PCR conditions have previously been optimized for a 4.7Kb product. Hence, these primer pair combinations are used to scan increasingly larger sections of the genome for deletions. If a mtDNA molecule exists bearing a deletion which lies within the confines of one or more of these primer pair combinations, a smaller PCR product will be generated, which should show a corresponding shift in size when the position of the H primer is altered. The presence of this deleted mtDNA molecule would then be confirmed by re-analyzing the mtDNA sample by part B of this method.

The second part of this method involves the addition of a second L strand primer, L480, which is situated 871bp to the left of the initial L567 primer (Diagram 3.3.2). This additional L strand primer creates an L strand primer shift, generating 4

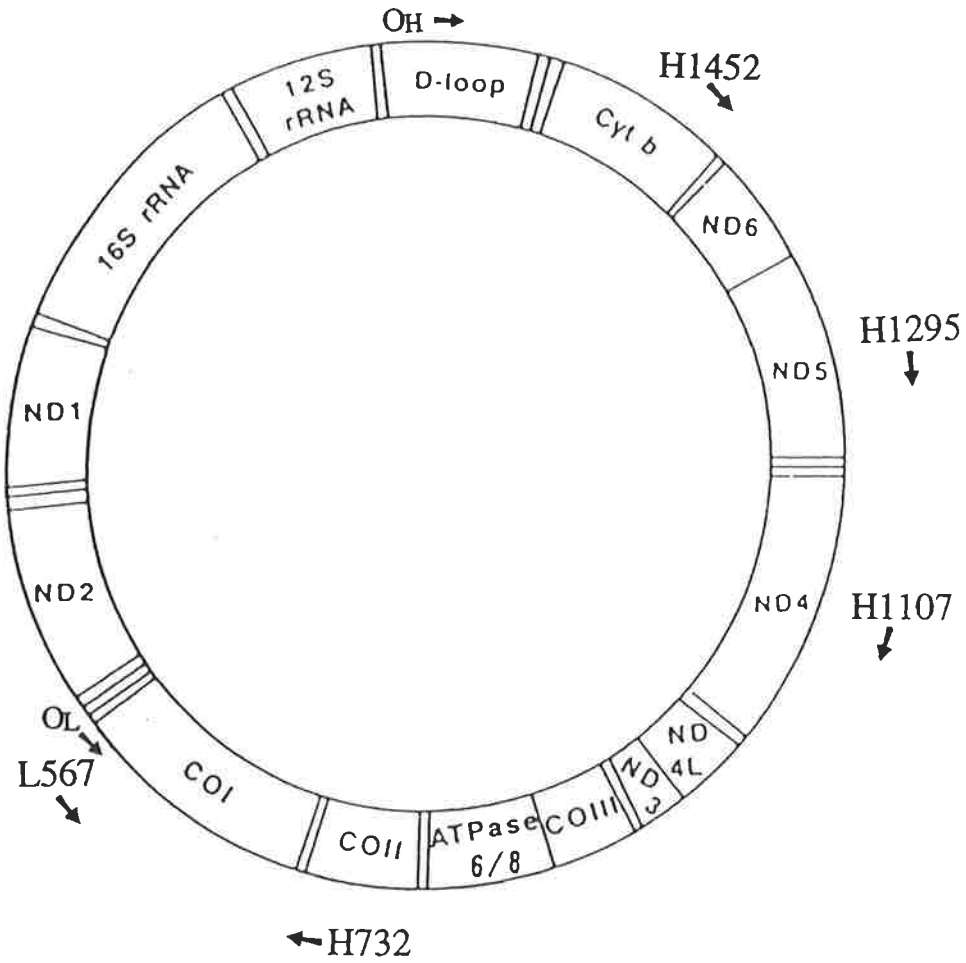


Diagram 3.3.1. A PCR method for analyzing the mouse mitochondrial genome for deletions:-part A

This diagram displays the genetic map of the mouse mitochondrial genome, showing the respective positions of the primers used within part A of the developed PCR method for detecting mtDNA deletions. This PCR analysis involves the following primer pair combinations; L567-H732, L567-H1107, L567-H1295 and L567-H1452 respectively, indicating a one-way, H strand PCR primer shift. The open bars represent tRNA (transfer RNA) genes. 12srRNA and 16sRNA are ribosomal RNA genes. ND1, ND2, ND3, ND4L, ND4, ND5 and ND6 genes code for subunits 1, 2, 3, 4L, 4, 5 and 6 of NADH dehydrogenase (Complex I). COI, COII and COIII genes code for subunits 1, 2 and 3 of Cytochrome c oxidase (Complex IV). The Cyt. b gene codes for Cytochrome b, a subunit of Ubiquinol-cytochrome c oxidoreductase (Complex III), and ATPase 6 and 8 genes code for 2 subunits of ATPase Synthase (Complex V). The light and heavy strand origins of replication are denoted as O_L and O_H respectively.

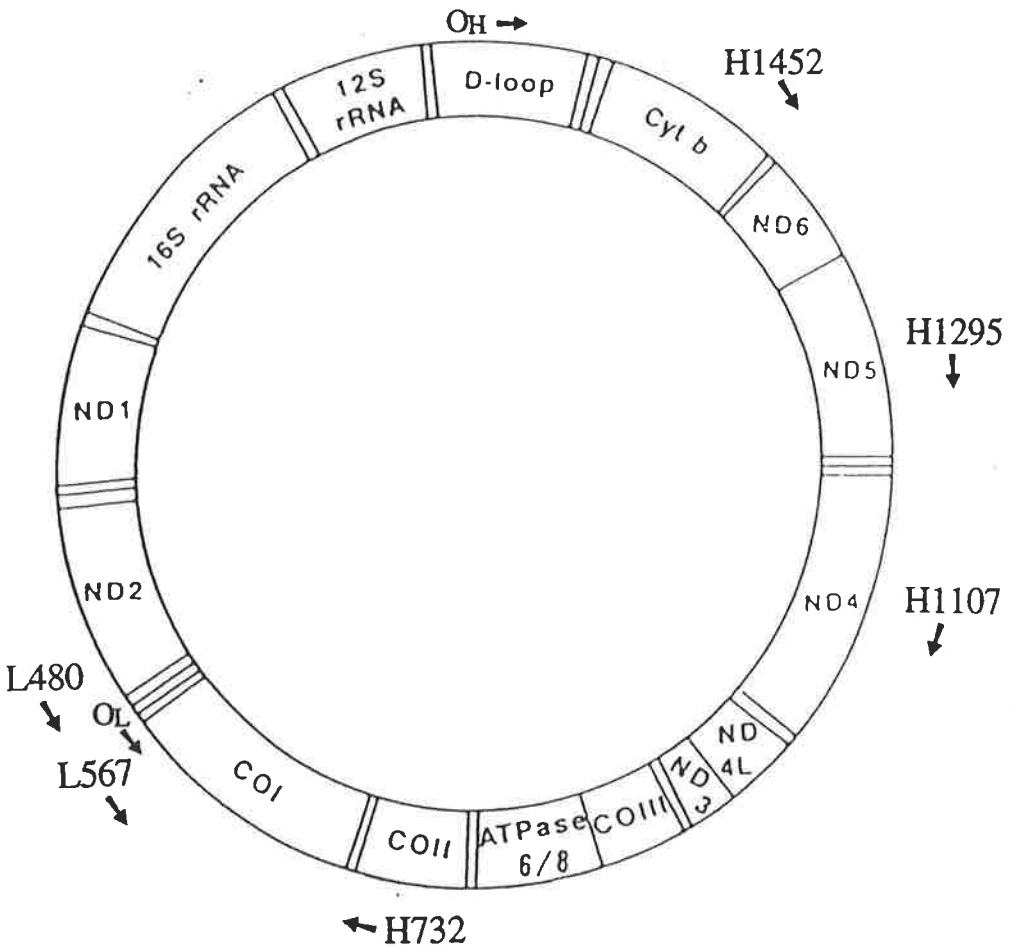


Diagram 3.3.2. A PCR method for analyzing the mouse mitochondrial genome for deletions:-part B

This diagram displays the genetic map of the mouse mitochondrial genome, showing the respective positions of the primers used within part B of the developed PCR method for detecting mtDNA deletions. This PCR analysis involves the following primer pair combinations; L567-H732, L480-H732, L567-H1107, L480-H1107, L567-H1295, L480-H1295, L567-H1452 and L480-H1452 respectively, indicating a two-way, L and H strand PCR primer shift. The open bars represent tRNA (transfer RNA) genes. 12srRNA and 16sRNA are ribosomal RNA genes. ND1, ND2, ND3, ND4L, ND4, ND5 and ND6 genes code for subunits 1, 2, 3, 4L, 4, 5 and 6 of NADH dehydrogenase (Complex I). COI, COII and COIII genes code for subunits 1, 2 and 3 of Cytochrome c oxidase (Complex IV). The Cyt. b gene codes for Cytochrome b, a subunit of Ubiquinol-cytochrome c oxidoreductase (Complex III), and ATPase 6 and 8 genes code for 2 subunits of ATPase Synthase (Complex V). The light and heavy strand origins of replication are denoted as O_L and O_H respectively.

additional PCR primer pair combinations of L480-H732, L480-H1107, L480-H1295 and L480-H1452 respectively. Therefore, in total there are 8 primer pair reactions involved within this second part of the method. This two way primer shift method distinguishes between authentic deleted PCR products and those bands which are non-specific PCR artifacts. Only those products which display a corresponding shift in size when both the L primer and the H primer are altered, will be considered to represent true deleted mtDNA PCR products. These bands will then be excised from the gel, re-amplified by the asymmetric PCR technique, and subsequently sequenced to determine the exact location of the deletion and the sequences which surround the end points of the deleted fragment.

Results:-

A typical gel, showing the results obtained when a 10 week old mouse liver mtDNA sample was analyzed by part A of the developed method, can be seen in Figure 3.3.1. The expected 1.6Kb fragment synthesized by the primers L567 and H732 was amplified as shown in lane 2. In addition to this known PCR product, an additional band of approximately 0.6Kb was also observed within this lane. The reaction involving the primer pair L567-H1107 (lane 3), resulted in a slight smearing pattern being observed, in contrast to the L567-H1295 PCR reaction (lane 4), where 3 bands of approximately 0.8Kb, 0.65Kb and 0.55Kb, were detected. No distinct products were generated by the primers L567 and H1452 from this mtDNA sample (lane 5). Overall, none of the synthesized products showed a corresponding increase in size when the H strand primer was shifted further away from the L567 primer (lanes 2-4 respectively).

This same mtDNA sample was subsequently analyzed by part B of the method for detecting deletions within the mouse mitochondrial genome (Figure 3.3.2). Again the expected PCR product of 1.6Kb was generated by the primers L567 and H732

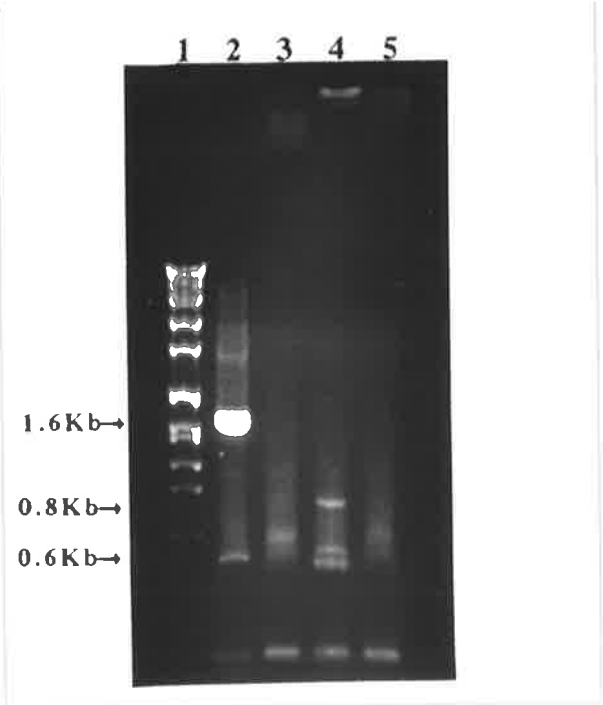


Figure 3.3.1. Analysis of mouse mtDNA by Part A of the developed PCR method: 10 week old mouse liver mtDNA was analyzed by Part A of the developed PCR method for detecting deletions within the mouse mitochondrial genome. This initial analysis involved the following primer pair combinations; L567-H732 (lane 2), L567-H1107 (lane 3), L567-H1295 (lane 4) and L567-H1452 (lane 5). Lane 1 corresponds to DNA size markers which range in size from 8.5Kb to 0.36Kb.

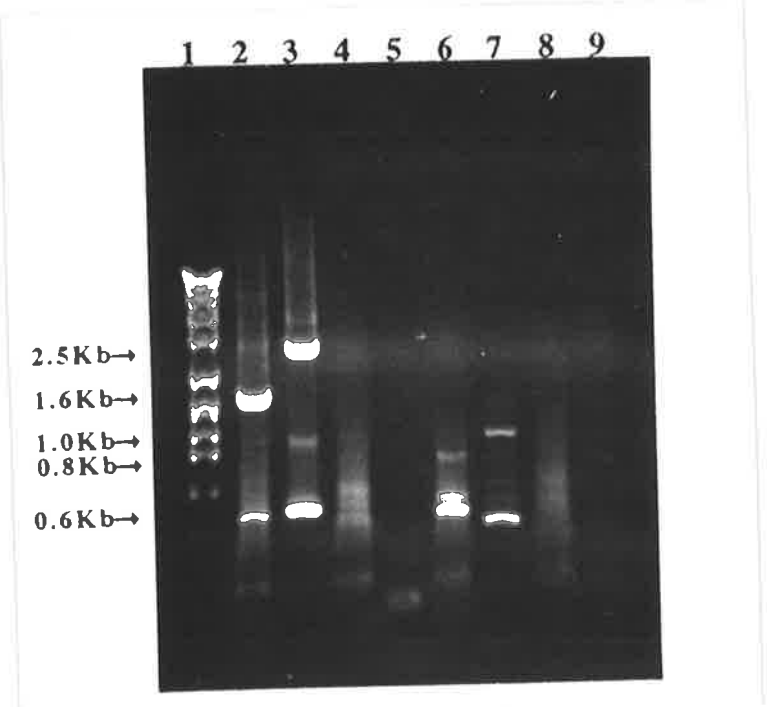


Figure 3.3.2. Analysis of mouse mtDNA by part B of the developed PCR method: 10 week old mouse liver mtDNA was analyzed by Part B of the developed PCR method for detecting deletions within the mouse mitochondrial genome. This second part of the method involved the following primer pair combinations; L567-H732 (lane 2), L480-H732 (lane 3), L567-H1107 (lane 4), L480-H1107 (lane 5), L567-H1295 (lane 6), L480-H1295 (lane 7), L567-H1452 (lane 8) and L480-H1452 (lane 9). Lane 1 corresponds to DNA size markers which range in size from 8.5Kb to 0.36Kb.

along with the smaller unknown band of approximately 0.6Kb (lane 2). Changing the L primer to L480, resulted in the amplification of a 2.5Kb product (lane 3), which corresponds to the expected PCR product. In addition two other smaller products of approximately 1.0Kb and 0.65Kb were also generated with these primers (lane 3). A streaking pattern was again observed with the primer pair L567-H1107 (lane 4), and changing the L primer to L480 resulted in no detectable bands being observed (lane 5).

The same 3 products of 0.8Kb, 0.65Kb and 0.55Kb were synthesized by the primers L567 and H1295 (lane 6), while 2 distinct bands of 1.0Kb and 0.5Kb were observed when the L primer was changed to L480 (lane 7). A streaking pattern was also observed with the primers L567 and H1452 (lane 8), and shifting the L primer to L480 resulted in no detectable PCR products being amplified (lane 9). Overall, the only band within this gel which displayed a primer shift pattern was the 1.6Kb fragment (lane 2), generated by the primers L567-H732, which showed a corresponding increase in size to 2.5Kb when the L primer was changed to L480 (lane 3).

Re-analysis of this mtDNA sample by part B of this method, using a hot start PCR protocol (Figure 3.3.3), resulted in a decline in the intensity of the smaller unknown PCR products, with a concomitant increase in the band intensity of the known expected PCR products of 1.6Kb and 2.5Kb (lanes 2 and 3 respectively). The 0.6Kb band previously generated by the primers L567 and H732 (lane 2), decreased in intensity such that it was virtually undetectable. The 1.0Kb product previously amplified by L480-H732 (lane 3) was no longer visible, while the 0.65 Kb band within this lane greatly decreased in intensity. By including this hot start PCR method, the smearing pattern previously generated by the primers L567 and H1107, became clearer identifying 4 faint bands with sizes of approximately 1.0Kb, 0.8Kb, 0.7Kb and 0.6Kb respectively (lane 4). In addition, a very faint product of 0.8Kb was generated by the primer pair L480-H1107 (lane 5) which was not previously observed. There was also a decline in the yield of the 3 products previously amplified by the primers L567 and H1295 in lane 6. With the primers L480 and H1295 (lane 7), the 2 bands of 1.0Kb and 0.5Kb decreased in intensity, along with the additional production of a faint larger

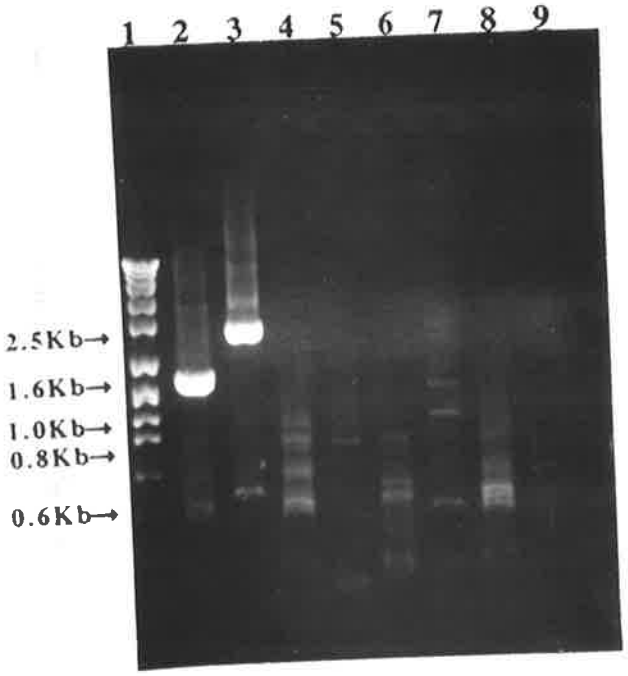


Figure 3.3.3. Analysis of mouse mtDNA by Part B of the developed PCR method, using a " Hot-Start" PCR protocol:
10 week old mouse liver mtDNA was analyzed by part B of the developed PCR method for detecting deletions within the mitochondrial genome, using a hot start PCR protocol. This analysis involved the following primer pair combinations; L567-H732 (lane 2), L480-H732 (lane 3), L567-H1107 (lane 4), L480-H1107 (lane 5), L567-H1295 (lane 6), L480-H1295 (lane 7), L567-H1452 (lane 8) and L480-H1452 (lane 9). Lane 1 corresponds to DNA size markers which range in size from 8.5Kb to 0.36Kb.

fragment of approximately 1.3Kb. The streaking pattern previously generated by the primers L567 and H1452 (lane 8) also became clearer revealing very faint bands of approximately 0.6Kb, 0.55Kb and 0.5Kb in size. No products were observed in lane 9 with the primers L480 and H1452. Again, the only product which showed a primer shift pattern, was the expected 1.6Kb fragment observed in lane 2, which displayed a corresponding increase in size to 2.5Kb, when the L primer was changed to L480 (lane 3).

Summary:-

A two part PCR method was developed for scanning the mouse mitochondrial genome for deletions within the major region between the 2 origins of replication. This method was based upon the principles of the primer shift PCR method, to detect and subsequently distinguish between authentic deleted mtDNA PCR products, and miscellaneous, non-specific PCR artifacts. The addition of a hot start PCR protocol to the PCR method appeared to decrease the number and intensity of non-specific PCR products while enhancing the intensity of the bands corresponding to the expected PCR products.

3.4 · PCR analysis of genetically engineered deleted mtDNA molecules:-

Experimental design:-

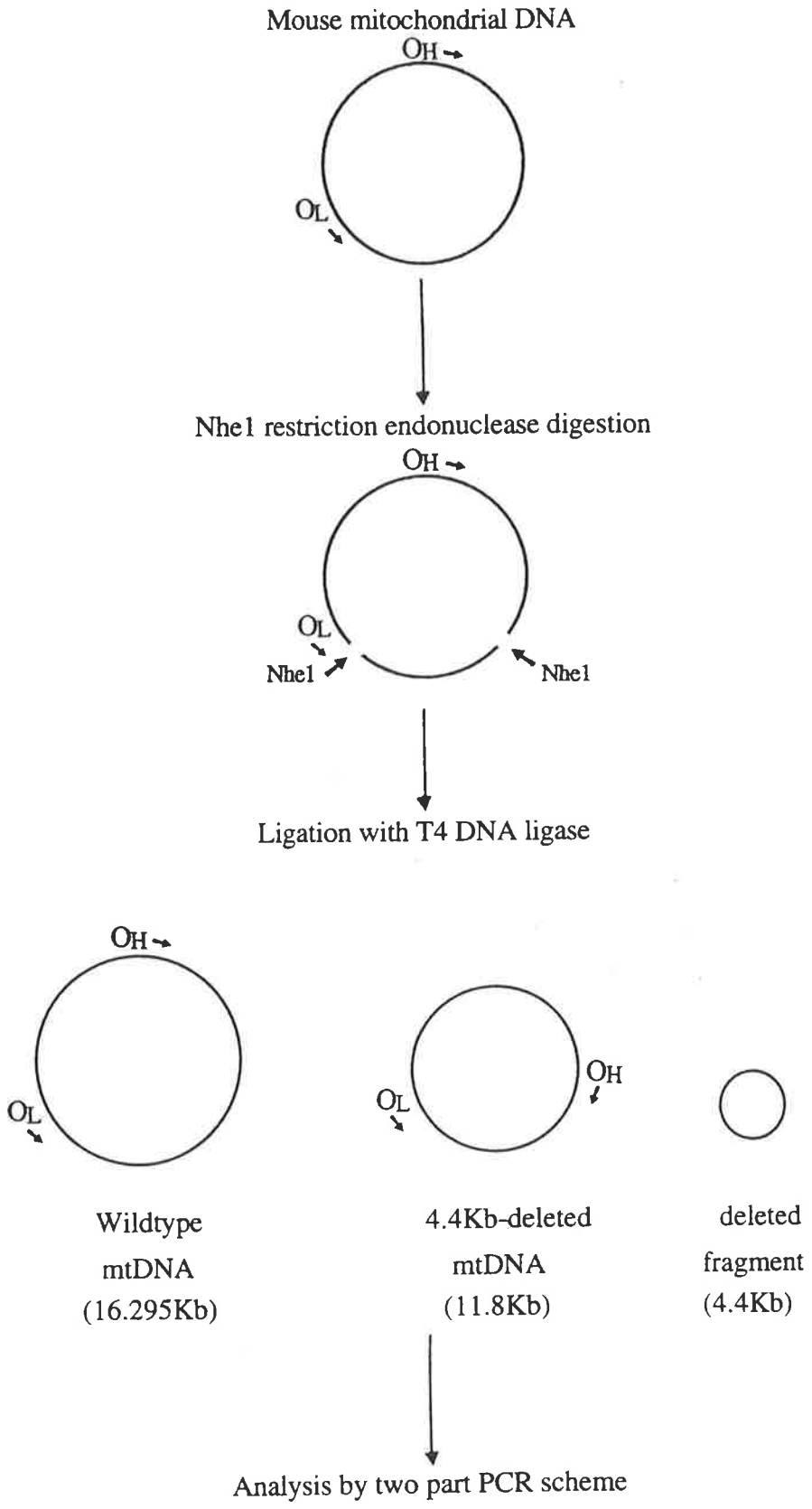
To verify that the developed two-part PCR method could detect mtDNA deletions within the mouse genome, it was tested against deleted mtDNA molecules

which had been genetically engineered by restriction endonuclease digestion and subsequent ligation reactions. An outline of this experiment can be seen in Diagram 3.4.0. Isolated mouse kidney mtDNA was digested with the restriction endonuclease Nhe 1, which cuts the genome at the nucleotide positions 5930 and 10395, generating two fragments of 11.830Kb and 4.465Kb. Following digestion, the entire DNA sample was purified and subsequently ligated via incubation with T4 DNA ligase. This should theoretically generate a heteroplasmic sample which contains molecules of 16.295Kb (wildtype mtDNA), 11.830Kb (mtDNA bearing a 4.465Kb deletion) and 4.465Kb (deleted fragment) respectively. Following purification and subsequent quantitation of the entire DNA sample, this mtDNA was analyzed by both part A and part B of the developed PCR method, to detect the presence of any mtDNA molecules bearing the 4.465Kb deletion.

The ratio of the amount of deleted mtDNA as compared to the wildtype genome (ie percentage deleted mtDNA) within this heteroplasmic sample was estimated by using the same semi-quantitative dilution PCR method used by Cortopassi & Arnheim (1990). This involved testing serial 1/10 dilutions of the heteroplasmic DNA sample with 2 sets of primers which were specific for either the wildtype (L567-H732) or the deleted mtDNA template (L480-H1107). These primer pair combinations would yield PCR products of 1.6Kb and 1.8Kb respectively. The ratio was calculated by comparing the limiting dilution required for the successful amplification of the normal PCR product of 1.6Kb, to the limiting dilution required for the successful amplification of the deleted PCR product of 1.8Kb.

The lowest percentage of this deleted mtDNA molecule that could be detected by the developed two-part PCR method, was estimated by preparing dilutions of the heteroplasmic sample with wildtype mtDNA of the same concentration. This would effectively decrease the amount of deleted mtDNA relative to the wildtype genome, whilst keeping the total amount of DNA within the reaction constant. These samples were examined by PCR analysis using the primers L567 and H1107, which should generate a deleted PCR product of 0.951Kb. The lowest % of deleted mtDNA which

Diagram 3.4.0. Genetic engineering of deleted mtDNA molecules:



could still be sufficiently amplified and visualized on an agarose gel was then taken as an estimate of the lowest % that could be detected by this PCR method.

Results:-

3.4.1. Selection of a restriction endonuclease to produce deleted mtDNA molecules:

Table 3.4.1 lists all the restriction endonucleases which cut the mouse mitochondrial genome at exactly 2 positions within the major region between the two origins of replication. Digestion with these restriction enzymes, followed by ligation would produce mtDNA molecules bearing deletions with sizes which range from 1.2Kb to 9.6Kb. A comparison of the sites where each of these restriction enzymes cut relative to the location of the PCR primers used within the previously developed PCR method for detecting deleted mtDNA molecules, is diagrammatically illustrated in the Diagram 3.4.1.

The restriction enzymes Aoc1, Bsu36I, Cvn1, Eco81I, Mst2, Sau1 and EcoRV, which are capable of generating deletions of greater than 9.4Kb, all cut the genome at positions which would delete the primer binding sites for the primers H732, H1107, H1295 and H1452. Therefore, the deletions generated by these restriction enzymes could not be detected by any of the primer pairs within this PCR method. Both the restriction enzymes AlwN1 and Nhe1 cut the genome at similar locations of (6026 and 10497) and (5930 and 10395) respectively, which would generate deletions of 4.471Kb and 4.465Kb respectively. These deletions could be detected by all the primer pairs involving the H primers H1107, H1295 and H1452, which would generate deleted PCR products ranging in size from approximately 0.95Kb to 5.2Kb.

Digestion with Pst1 would generate a 3.812Kb deletion which could be detected by all the primer pairs involving the H primers H1295 and H1452, which would generate deleted PCR products which range in size from 3.4Kb to 5.9Kb. The 2.44Kb

Table 3.4.1 Restriction endonucleases which cut the mouse mtDNA genome twice between the positions 5160 and 16295: This table summarizes all the restriction endonucleases which cut the mouse mtDNA genome at 2 sites within the major region between the two origins of replication. The nucleotide position where each enzyme cuts is listed, and is numbered according to the mouse mtDNA sequence derived by Bibb et al (1981). The size (kilobases) of the deletion which would be produced by the digestion of mouse mtDNA with each enzyme is also shown.

Restriction Endonuclease:	Cut Positions:		Deletion:
Aoc1	6350	16022	9.672Kb
Bsu36I	"	"	"
Cvn1	"	"	"
Eco81I	"	"	"
Mst2	"	"	"
Sau1	"	"	"
EcoRV	5369	14822	9.453Kb
AlwN1	6026	10497	4.471Kb
Nhe1	5930	10395	4.465Kb
Pst1	8424	12236	3.812Kb
Asu2	5220	7668	2.448Kb
Aqu1	11194	13551	2.357Kb
Ava1	"	"	"
BspM1	12945	14210	1.265Kb

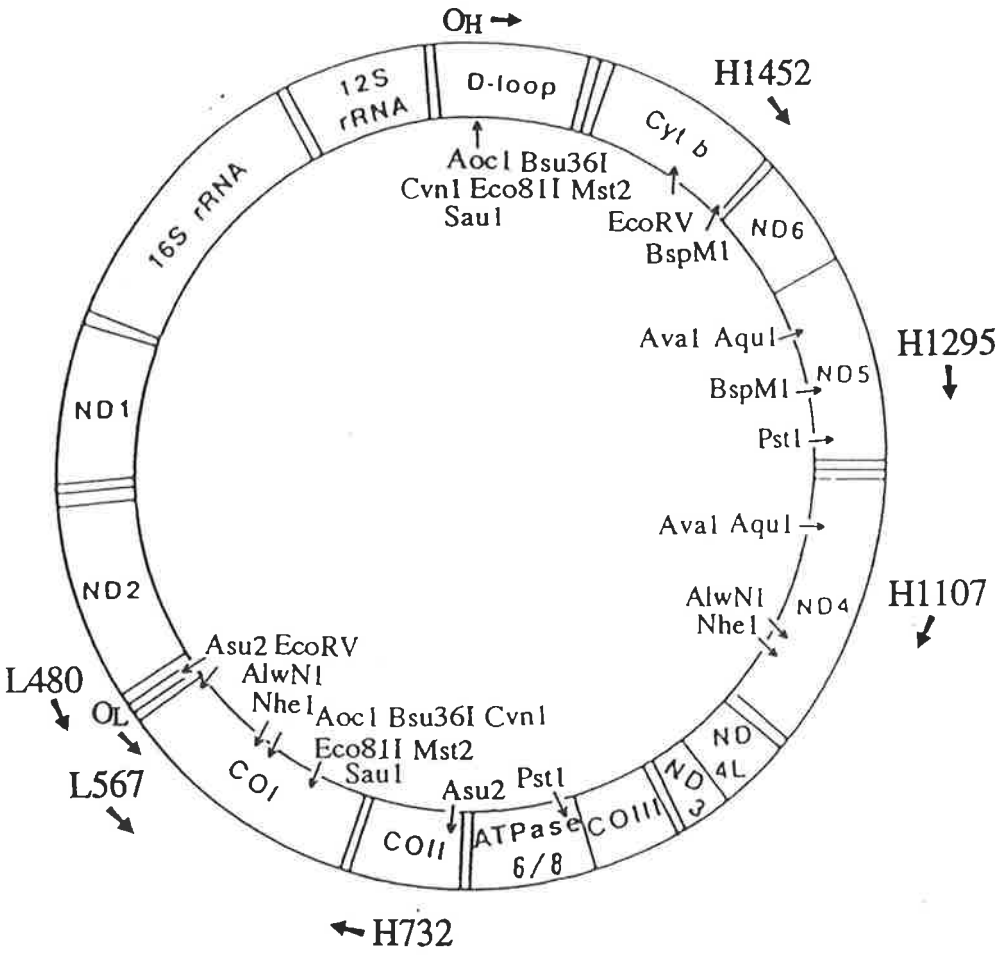


Diagram 3.4.1. Restriction endonucleases which cut the mouse mtDNA genome twice between the positions 5160 and 16295:

This diagram shows the genetic map of the mouse mtDNA genome, highlighting all the restriction endonucleases which cut the genome twice within the major region between the two origins of replication. This map also displays the respective positions of the PCR primers utilized in the previously developed two-part PCR method for detecting deletions, relative to the position of each of these restriction enzyme cutting sites. The open bars represent tRNA (transfer RNA) genes. 12srRNA and 16sRNA are ribosomal RNA genes. ND1, ND2, ND3, ND4L, ND4, ND5 and ND6 genes code for subunits 1, 2, 3, 4L, 4, 5 and 6 of NADH dehydrogenase (Complex I), COI, COII and COIII genes code for subunits 1, 2 and 3 of Cytochrome c oxidase (Complex IV), the Cyt. b gene codes for Cytochrome b, a subunit of Ubiquinol-cytochrome c oxidoreductase (Complex III), and ATPase 6 and 8 genes code for 2 subunits of ATPase Synthase (Complex V). The light and heavy strand origins of replication are denoted as O_L and O_H respectively.

deletion produced by digestion with Asu2, could be detected by the primer pairs L480-H1107, L480-H1295 and L480-H1452, which would generate deleted PCR products ranging in size from 3.8Kb to 7.3Kb. Aqu1, Ava1 and BspM1 would all create deletions which could only be detected by the primer pairs involving the H primer H1452, generating deleted PCR products of greater than 6.5Kb.

Reviewing all of these endonucleases and bearing in mind that 5Kb was the largest product which could be successfully amplified, the restriction enzyme Nhe 1 was selected to generate deletions within the mouse mitochondrial genome since it would produce a 4.4Kb deletion which could be detected by numerous primer pair combinations of the two part PCR method, generating deleted PCR products of approximately 5Kb or less.

3.4.2. Genetic engineering of deleted mtDNA molecules:

Prior to restriction endonuclease digestion, an aliquot of 10 week old mouse kidney mtDNA was run on a 1% agarose gel, as shown in lane 2 of Figure 3.4.2. As can be seen, the DNA sample did not migrate through the gel, resulting in the band corresponding to mtDNA being retained within the loading well. A faint smearing pattern was also observed throughout this lane. After this same DNA sample was digested with the restriction enzyme Nhe1, 2 expected bands of approximately 11.8Kb and 4.4Kb were observed (lane 3), which indicates successful digestion of the genome. However, subsequently incubating this DNA sample with T4 DNA ligase resulted in these two bands no longer being detected, and again the DNA sample was retarded within the loading well of the gel (lane 4).

3.4.3. PCR analysis of genetically engineered deleted mtDNA molecules:

10 week old mouse kidney mtDNA which had been digested with the restriction enzyme Nhe 1 and subsequently ligated, was analyzed by the previously developed

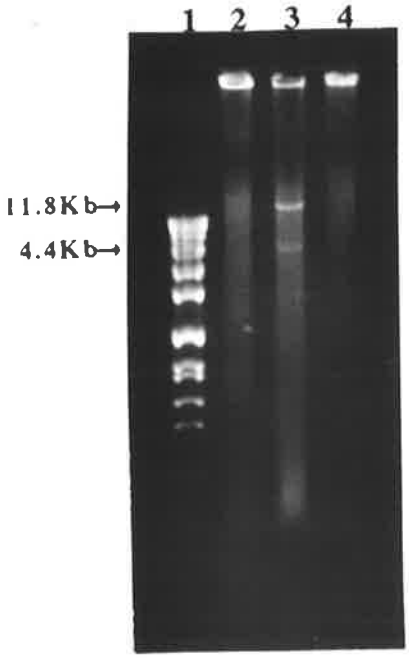


Figure 3.4.2. Genetic engineering of deleted mtDNA molecules:
10 week old mouse kidney mtDNA (lane 2) was digested with the restriction endonuclease Nhe1 (lane 3), and subsequently ligated by incubation with T4 DNA ligase (lane 4). Approximately 2μg of mtDNA was loaded within each well. DNA size markers were run in lane 1 with sizes which ranged from 8.5Kb to 0.36Kb.

two part PCR method. This mtDNA sample was initially scanned for the presence of DNA molecules bearing a 4.465Kb deletion by part A of the developed method (Figure 3.4.3a). Using the primer pair L567-H732 (lane 2), a definite product of 1.6Kb was synthesized, which corresponds to the expected PCR product amplified from the wildtype, non-deleted mtDNA genome. In lane 3, a 0.95Kb product was generated by the primers L567 and H1107. This PCR product corresponds in size to the deleted PCR product which would be produced by these primers annealing to the 4.4Kb-deleted template. Further evidence to support that deleted mtDNA molecules existed within this DNA sample was obtained by shifting the H primer to H1295, where a corresponding shift in size of this 0.95Kb product to 2.8Kb was observed (lane 4). Further shifting the primer to H1452, resulted in a further increase in the size of the deleted PCR product to yield a faint band of 4.4Kb (lane 5).

The presence of deleted mtDNA molecules existing within this DNA sample was confirmed by performing part B of the PCR method on this mtDNA sample (Figure 3.4.3b). Again, the 1.6Kb band due to amplification of the wildtype DNA template was generated by the primer pair L567-H732 (lane 2). This PCR product displayed a corresponding increase in size when the L primer was shifted to L480, synthesizing a 2.5Kb product (lane 3). The 0.95Kb deleted PCR product amplified by the primers L567 and H1107 (lane 4), also showed this same primer shift pattern when the L primer was changed to L480, generating a 1.8Kb deleted PCR product (lane 5). Again, shifting the H primer to H1295 also resulted in a corresponding increase in size of the 0.95Kb product to yield a product of 2.8Kb (lane 6). This 2.8Kb product also displayed a corresponding increase in size when the L primer was changed to L480, with a 3.7Kb band being observed (lane 7). No PCR product bands were generated by the primer pairs involving the H1452 primer (lanes 8 and 9). These results confirm that the DNA sample examined was heteroplasmic, containing both wildtype and 4.4Kb-deleted mtDNA molecules.

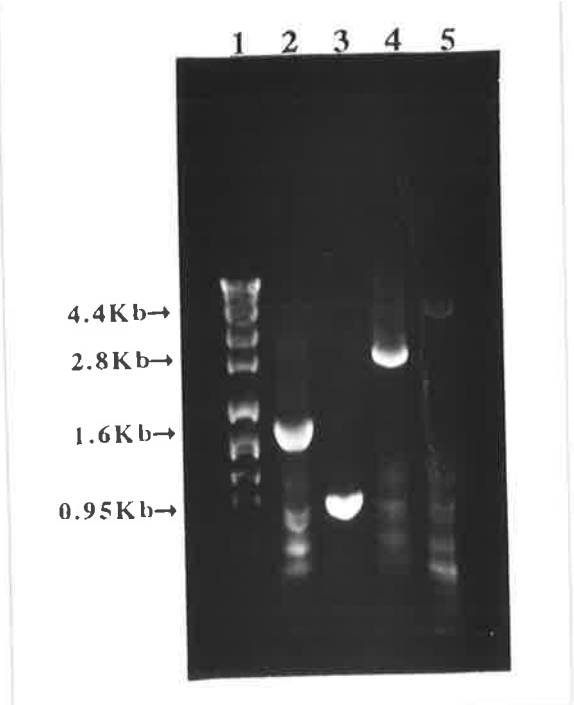


Figure 3.4.3a. Part A PCR analysis of Nhe1 digested mtDNA:
10 week old mouse kidney mtDNA, which had previously been digested with Nhe1 and subsequently ligated, was analyzed by part A of the method for detecting deletions. The primer pairs were as follows; L567-H732 (lane 2), L567-H1107 (lane 3), L567-H1295 (lane 4) and L567-H1452 (lane 5). Lane 1 corresponds to DNA size markers which range in size from 8.5Kb to 0.36Kb.

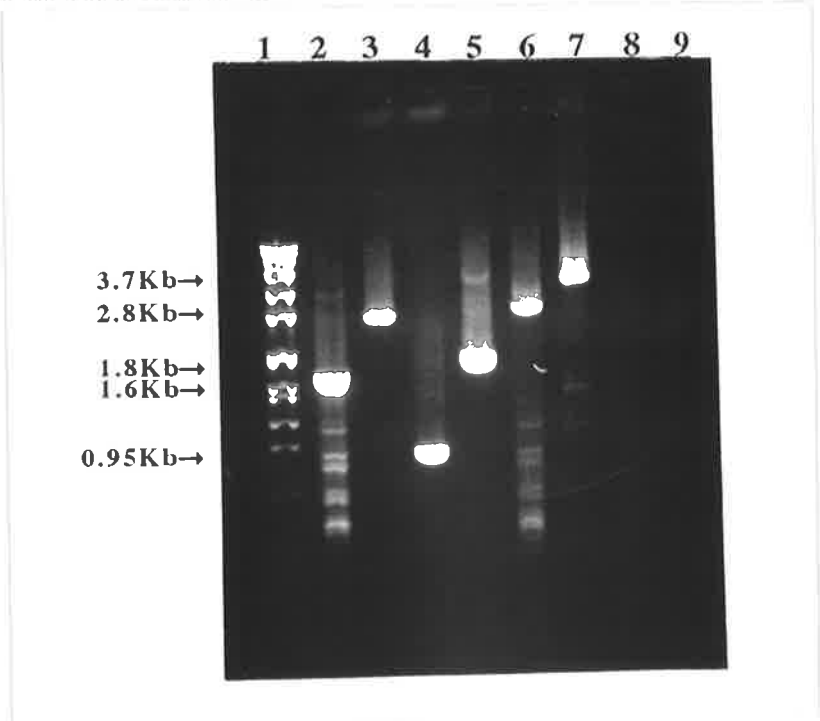


Figure 3.4.3b. Part B PCR analysis of Nhe1 digested mtDNA:
10 week old mouse kidney mtDNA, which had previously been digested with Nhe1 and subsequently ligated, was analyzed by part B of the method for detecting deletions. The primer pairs were as follows; L567-H732 (lane 2), L480-H732 (lane 3), L567-H1107 (lane 4), L480-H1107 (lane 5), L567-H1295 (lane 6), L480-H1295 (lane 7), L567-H1452 (lane 8) and L480-H1452 (lane 9). Lane 1 corresponds to DNA size markers which range in size from 8.5Kb to 0.36Kb.

3.4.4. Estimation of the ratio of deleted mtDNA : wildtype mtDNA:

From serial 1/10 dilutions of a 10 week old mouse kidney mtDNA sample which had been digested with the restriction enzyme Nhe1 and subsequently ligated, the wild type genome was amplified by the primers L567 and H732, and the 4.4Kb-deleted mtDNA was detected by the primer pair L480-H1107. These reactions produced PCR products of 1.6Kb and 1.8Kb respectively. PCR products of 1.6Kb could only be detected up to a 1 in 1000 dilution of the heteroplasmic DNA sample added to the PCR reaction (Figure 3.4.4a, lanes 2-5). This same limiting dilution of 1 in 1000 was also found for amplifying the 1.8Kb deleted PCR product by the primers L480 and H1107 from the deleted mtDNA templates (Figure 3.4.4b, lanes 2-5), which indicates that approximately equal amounts of wildtype and deleted mtDNA exist within this heteroplasmic DNA preparation, implying that the ratio of deleted mtDNA : wildtype DNA is approximately 1:1.

3.4.5. Estimation of the lowest % deleted mtDNA detectable by the PCR method:

The lowest percentage of deleted mtDNA molecules which could be detected by the developed PCR method was determined by diluting the amount of deleted mtDNA within the heteroplasmic mtDNA sample with wildtype mtDNA. The results of this experiment are shown in Figure 3.4.5, where the primers L567 and H1107 were utilized to amplify the 4.4Kb-deleted mtDNA molecules. Decreasing the percentage of deleted mtDNA in the reaction mixture whilst keeping the total amount of DNA in the reaction constant, resulted in a decline in the amount of the 0.95Kb deleted PCR product being synthesized. Decreasing the percentage from 50% to 0.01% (lanes 2-7 respectively), significantly decreased the band intensity of the 0.95Kb product, and further decreasing this level resulted in no deleted PCR product being detected, with a concomitant increase in the number and intensity of non-specific PCR products being observed (lanes 8-10). This data suggests that the PCR method employed could detect deleted mtDNA molecules at concentrations of 1 in 10 000 relative to the wildtype

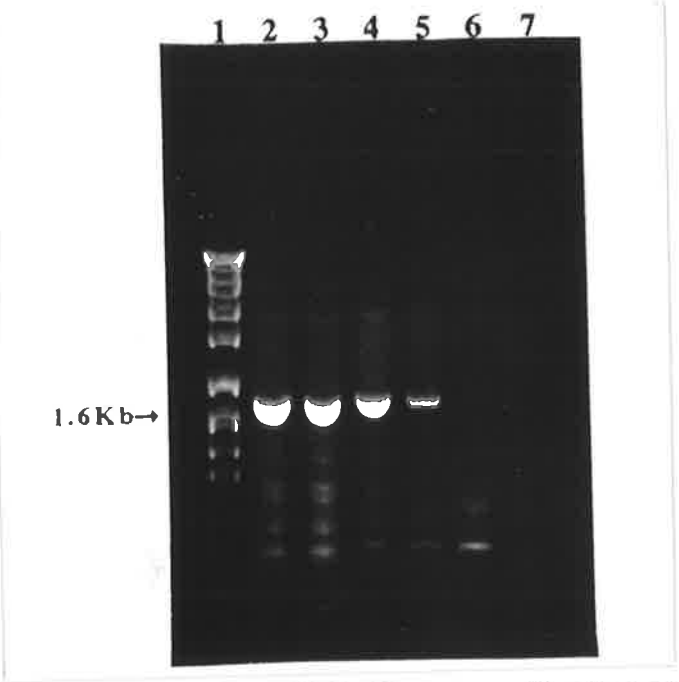


Figure 3.4.4a. The limiting dilution which allows amplification of the L567-H732 product:
A mixture of wildtype and deleted mouse mtDNA, synthesized by previous genetic engineering experiments was serially diluted 1/10, and was subsequently analyzed by the primer pair L567-H732 for the amplification of the 1.6Kb wildtype PCR product. The dilutions were as follows; undiluted (lane 2), 1/10 (lane 3), 1/100 (lane 4), 1/1000 (lane 5), 1/10 000 (lane 6) and 1/100 000 (lane 7). DNA size markers were run in lane 1 which range in size from 8.5Kb to 0.36Kb.

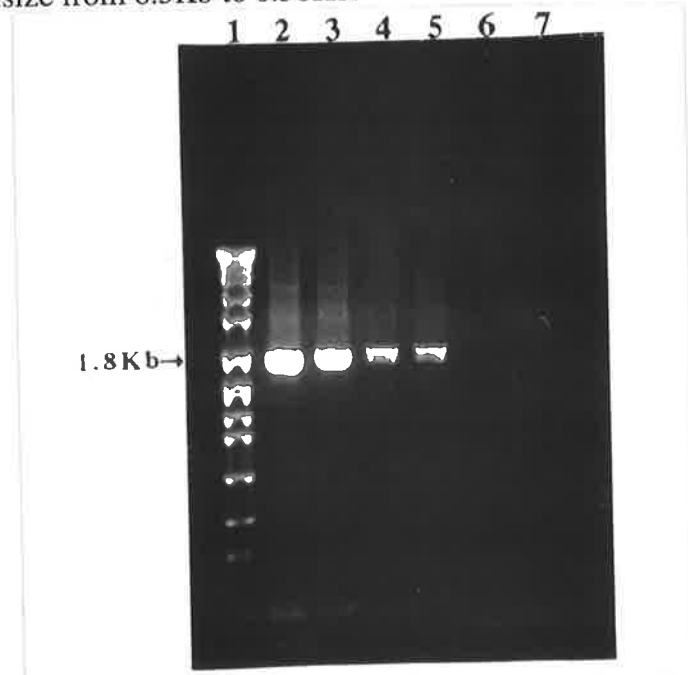


Figure 3.4.4b. The limiting dilution which allows amplification of the deleted L480-H1107 product:
A mixture of wildtype and deleted mtDNA, synthesized by previous genetic engineering experiments was serially diluted 1/10, and was subsequently analyzed by the primer pair L480-H1107 for the amplification of the deleted 1.8Kb PCR product. The dilutions were as follows; undiluted (lane 2), 1/10 (lane 3), 1/100 (lane 4), 1/1000 (lane 5), 1/10 000 (lane 6) and 1/100 000 (lane 7). DNA size markers were run in lane 1 which ranged in size from 8.5Kb to 0.36Kb.

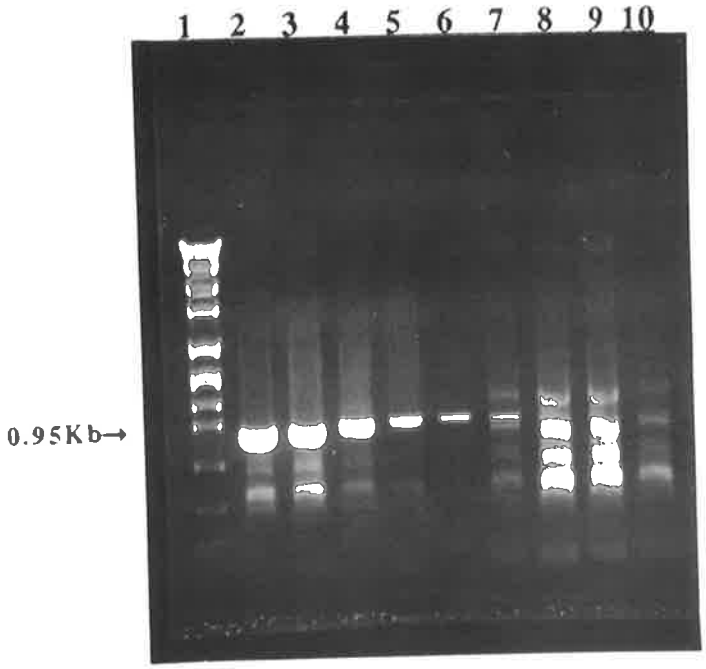


Figure 3.4.5. Estimation of the lowest % of deleted mtDNA detectable by the developed PCR scheme:
The % of deleted mtDNA within a DNA sample ,which was obtained from previous genetic engineering experiments, was decreased by performing dilutions with wildtype DNA of the same concentration. The primer pair L567-H1107 was used to amplify the deleted genomes in reactions which contained; 50% , 25%, 10%, 1%, 0.1%, 0.01%, 0.001%, 0.0001% and 0.00001% deleted mtDNA (lanes 2-10 respectively). DNA size markers with sizes which range from 8.5Kb to 0.36Kb were run in lane 1.

mitochondrial genome population.

Summary:-

Mitochondrial DNA molecules bearing a 4.4Kb deletion, produced by the Nhe1 restriction enzyme digestion of a 10 week old mouse kidney mtDNA sample, were found to be successfully detected by both parts of the developed PCR method designed for detecting deletions within the mouse mitochondrial genome. The percentage of deleted mtDNA molecules within this heteroplasmic DNA sample preparation was estimated to be approximately 50%, while the lowest percentage of deleted mtDNA molecules which could be detected by this PCR method was calculated to be approximately 0.01%.

3.5. Conclusions:-

Within this chapter, a PCR method specific for the mouse mitochondrial genome was developed. From analyzing the position and sequences of PCR primers which have previously been used for amplifying the human mitochondrial genome, both H (heavy) and L (light) strand primers of 18-22 nucleotides in length, located within protein coding genes, were designed for the mouse mitochondrial genome. Using standard PCR conditions, the largest product which could be successfully amplified was a 4.7Kb fragment, which gave a faint band when visualized by agarose gel electrophoresis. Following optimization of all the PCR reaction conditions, the intensity of this 4.7Kb band was significantly enhanced, and all the designed mouse

PCR primers were found to be capable of annealing to the template and generating PCR products under these optimized conditions.

A two part PCR method was subsequently developed for scanning the major region between the two origins of replication of the mouse mitochondrial genome, for the presence of deletions. This method was validated by analyzing deleted mtDNA molecules which had been genetically engineered by restriction endonuclease and subsequent ligation reactions. The lowest percentage of these deleted mtDNA molecules which could be significantly amplified by this PCR method and detected on an agarose gel was calculated to be approximately 0.01%.

Chapter 4:

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

4.0. Introduction:-

Within this chapter, mitochondrial DNA from various aged mice tissues (10 weeks old, 19 months old and 42 months old) was examined for the presence of age-related deletions, within the major region between the two origins of replication. Four tissues from each age group were examined which included the brain, heart, skeletal muscle and liver. To detect deleted mtDNA molecules within these tissues, each sample was analyzed by the two part PCR method previously described in chapter 3, which was designed specifically for the mouse mitochondrial genome.

Each tissue was initially examined for the presence of mtDNA molecules bearing deletions by part A of the developed PCR method. Following the PCR analysis of each sample, the sizes of the products generated by each primer pair combination were calculated and examined to determine if these products showed a corresponding shift in size when the position of the H strand primer was changed.

To confirm the presence of any deleted PCR products generated by part A, the brain mtDNA samples were subsequently analyzed by part B of this method, where the size of each synthesized product was calculated when both the L and the H primers were shifted. Only those DNA bands which showed a corresponding shift in size when both the L and H primers were individually altered, were considered to represent PCR products generated from deleted mtDNA templates.

Following the analysis of all the samples within each age group, the size and number of DNA products synthesized by each primer pair combination were compared

to determine if any of the products were age dependent. The DNA banding patterns observed for each different tissue were then subsequently analyzed to determine if any of these generated products were tissue specific.

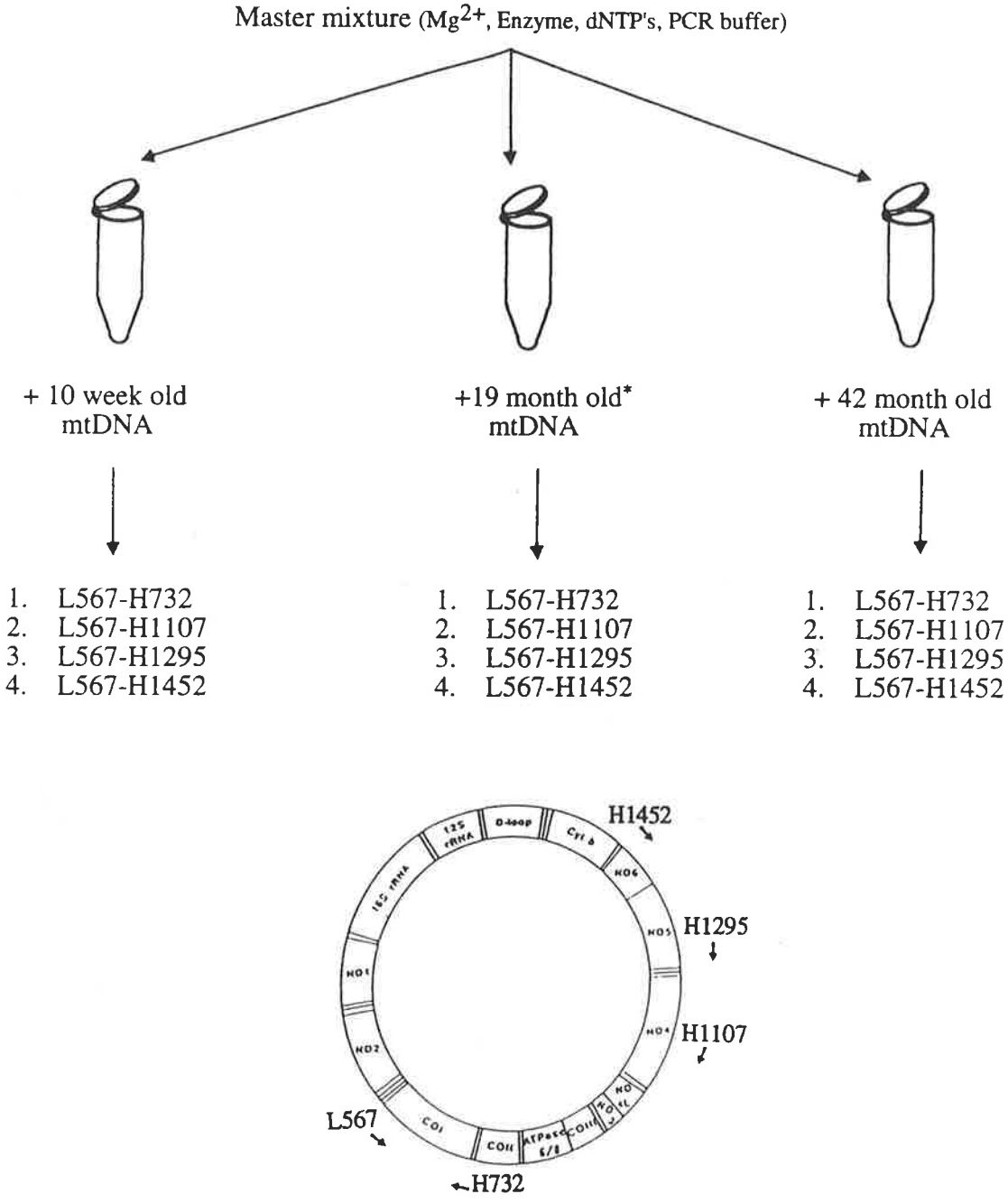
4.1. Analysis of various aged mice tissues by Part A of the developed PCR method:-

Experimental design:-

Initially each tissue (brain, heart, skeletal muscle and liver) was analyzed for the presence of age-related deleted mtDNA by part A of the developed PCR method, which involved the following primer pair combinations; L567-H732, L567-H1107, L567-H1295 and L567-H1452. The brain was the first tissue to be analyzed, where at least one mtDNA sample from each of the 3 age groups was examined at the same time. This involved preparing a master mixture of all the PCR reaction reagents (omitting the template DNA and the primers), and subsequently aliquoting this into 3 reaction tubes (see Diagram 4.1). The desired DNA template corresponding to each age group was then added to one of each of these 3 tubes. The mixture within each tube was then aliquoted into 4 separate reaction tubes which each contained a different primer pair combination of part A of the developed PCR method. All the reaction tubes were then placed within the DNA thermal cycling machine together, and an aliquot of each reaction was subsequently run on the same agarose gel.

Within each age group, the sizes of the products generated by each primer pair combination were calculated, and examined to determine if any particular product displayed a corresponding shift in size when the position of the H strand primer was altered. The results obtained from each age group were then compared to determine if any of the observed bands were age dependent. This procedure was repeated for each

Diagram 4.1. Experimental protocol for analyzing different aged mice tissues by part A of the developed PCR method:



All 12 PCR reactions were placed within the thermal cycling machine together, and an aliquot of each reaction was subsequently run on an agarose gel.

* Heart and muscle samples of this age group were unavailable for analysis due to -80°C freezer malfunction.

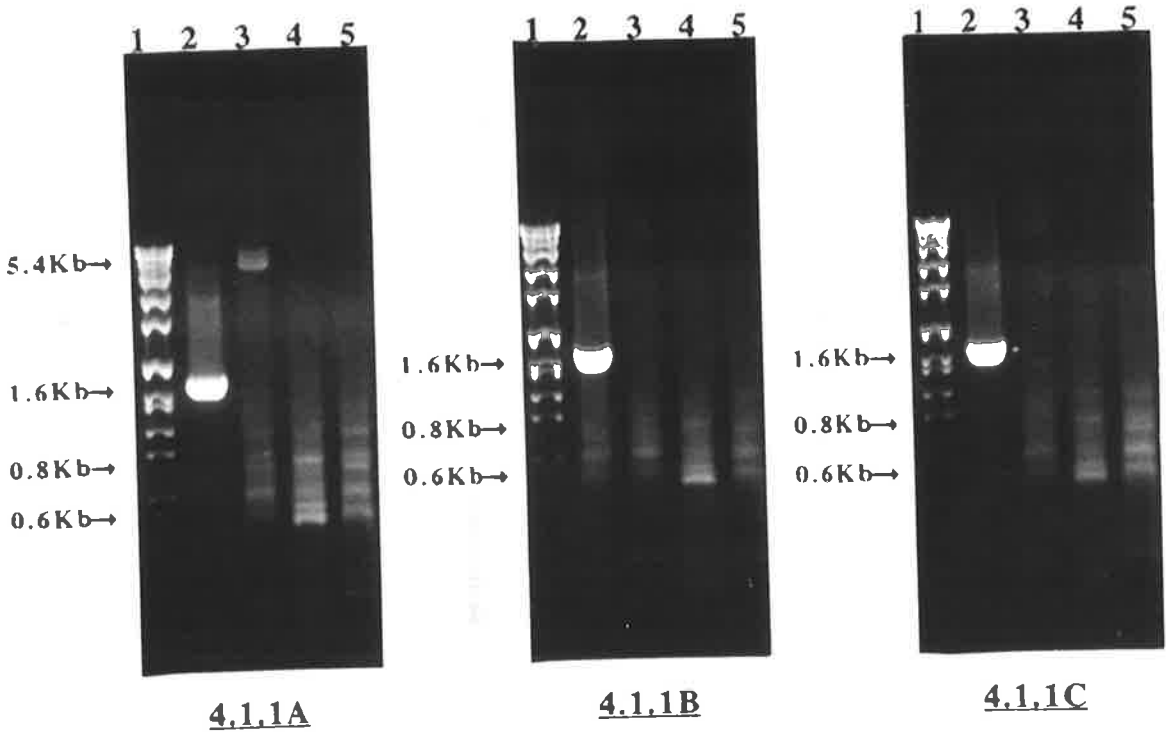
of the different mouse tissues, and the size and number of products amplified from each tissue were then compared to determine if any of the products were tissue specific.

Results:-

4.1.1. Analysis of Brain mtDNA by part A of the developed PCR method:

Representative gels showing the results obtained when a 10 week old, a 19 month old and a 42 month old mouse brain mtDNA sample, were analyzed by part A of the developed PCR method, are shown in Figures 4.1.1A, B and C respectively. These samples were analyzed on the same day using identical reaction conditions. PCR analysis with the primer pair L567-H732, resulted in the expected 1.6Kb PCR product being amplified from each of the DNA samples, irrespective of their age (lane 2 in each figure). A faint band of approximately 0.7Kb was also observed within this lane for the 19 month old brain sample (Figure 4.1.1B, lane 2). Analysis with the primers L567 and H1107 resulted in a 0.7Kb product being synthesized from each of the mtDNA samples (lane 3 in each figure), while the expected PCR product of 5.4Kb was only generated from the 10 week old sample (Figure 4.1.1A, lane 3). Three bands of 0.8Kb, 0.65Kb and 0.55Kb were observed when each sample was analyzed by the primer pair L567-H1295 (lane 4 of each figure), and shifting the H strand primer to H1452, resulted in the synthesis of four products of 1.0Kb, 0.8Kb, 0.7Kb and 0.6Kb being generated from each of the different aged mtDNA samples (Figures 4.1.1A, B & C, lane 5).

Overall, the only band which displayed a H strand primer shift pattern within these 3 figures, was the 1.6Kb product amplified from the 10 week old mtDNA sample by the primers L567 and H732 (Figure 4.1.1A, lane 2). This expected PCR product of 1.6Kb showed a corresponding increase in size to 5.4Kb, when the H strand primer was shifted to the nucleotide position 11072 (lane 3).



Figures 4.1.1A, B & C. Analysis of Brain mtDNA by part A of the developed PCR method:
Brain mtDNA from a 10 week old, a 19 month old and a 42 month old mouse were analyzed by part A of the previously developed PCR method (Figures 4.1.1A, B & C respectively). This involved the analysis of each sample by the following primer pair combinations; L567-H732, L567-H1107, L567-H1295 and L567-H1452 (lanes 2, 3, 4 and 5 respectively within each figure). DNA size markers ranging from 8.5Kb to 0.36Kb were run in lane 1 of each figure.

As previously mentioned, these figures only represent the analysis of one brain mtDNA sample from each age group, and the banding pattern derived from each sample varied slightly. A summary of the products generated from all the brain samples within each age group is shown in the Graphs 4.1.1A-D, which correspond to the different primer pair combinations. In Graph 4.1.1A, which represents the results obtained with the primer pair L567-H732, the number of brain mtDNA samples which resulted in the amplification of a particular size product is expressed as a percentage of the total number of DNA samples examined within that age group. As can be seen, 100% of all the brain mtDNA samples examined within each age group, displayed the expected 1.6Kb band. However, a smaller product of 0.6Kb was only amplified from 30, 40 and 22.2% of the 10 week old, 19 month old and the 42 month old mtDNA samples examined respectively, while a 0.7Kb product was generated from approximately the same number of samples examined within each age group.

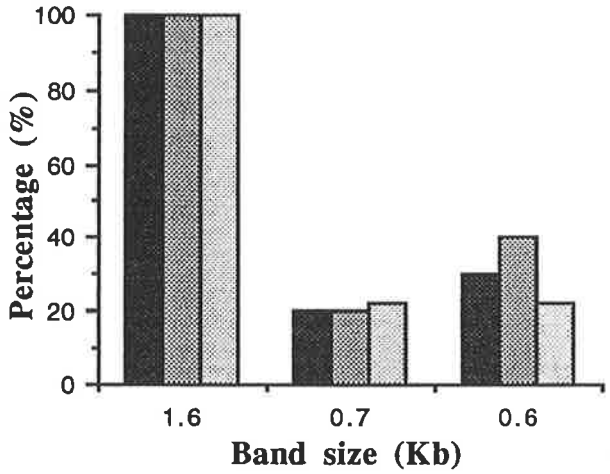
With the primer pair L567-H1107 (Graph 4.1.1B), a total of 5 different DNA products were amplified with approximate sizes of 5.4Kb, 1.0Kb, 0.8Kb, 0.7Kb and 0.6Kb. These bands were observed following the analysis of samples within each age group. The expected 5.4Kb PCR product was generated from 30, 40 and 22.2% of the 10 week old, the 19 month old and the 42 month old mtDNA samples examined respectively, while the percentage of 10 week old mtDNA samples which generated the 1.0Kb (60%), 0.8Kb (60%), 0.7Kb (30%) and the 0.6Kb (50%) products was greater than or equal to the corresponding percentages of the older two age groups.

Examination by the primers L567 and H1295, resulted in a total of four different bands being observed with sizes of approximately 1.0Kb, 0.8Kb, 0.65Kb and 0.55Kb (Graph 4.1.1C). These products were amplified following the analysis of DNA samples from each age group, where the percentage of 10 week old samples which resulted in the synthesis of these products (40%, 60%, 60% and 60% respectively), was greater than or equal to the respective percentages of the older two age groups.

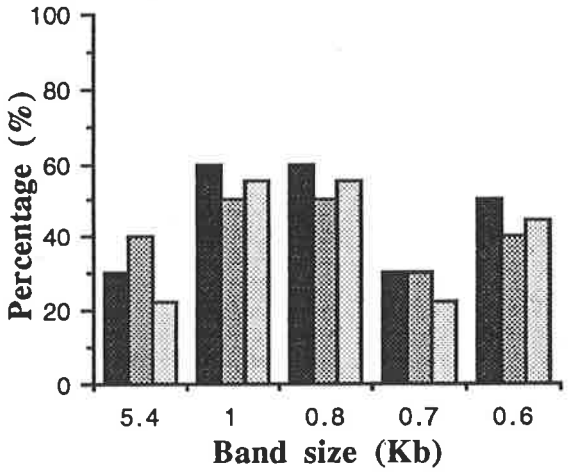
Graphs 4.1.1A, B, C & D. Analysis of Brain mtDNA by part A of the developed PCR method:

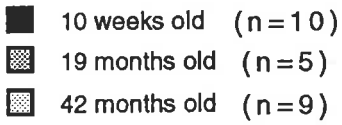
These graphs display the size (kilobases) of the products amplified from various aged mouse brain mtDNA samples (10 weeks old, 19 months old and 42 months old), by each primer pair combination of part A of the developed PCR method. This analysis involved the following primer pairs; L567-H732, L567-H1107, L567-H1295 and L567-H1452, with the results shown in the graphs 4.1.1A, B, C & D respectively. The number of brain mtDNA samples which resulted in the amplification of a particular product is expressed as a % of the total number of DNA samples examined within that age group.

Graph 4.1.1A. L567-H732

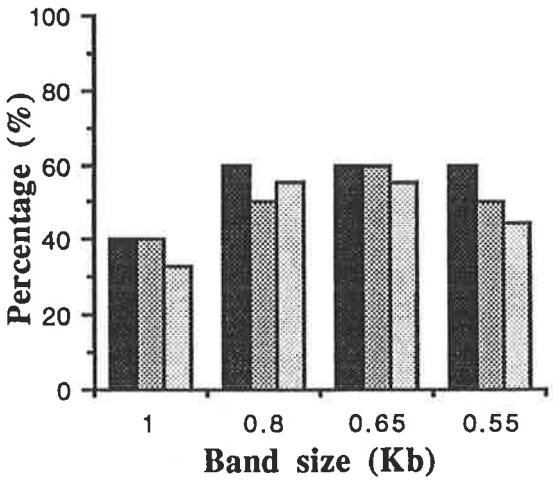


Graph 4.1.1B. L567-H1107

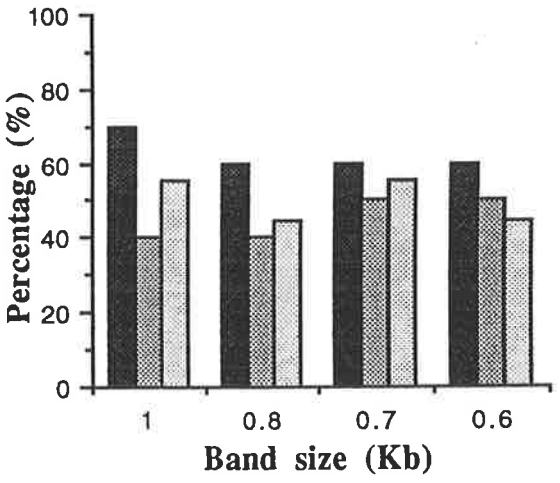




Graph 4.1.1C. L567-H1295



Graph 4.1.1D. L567-H1452

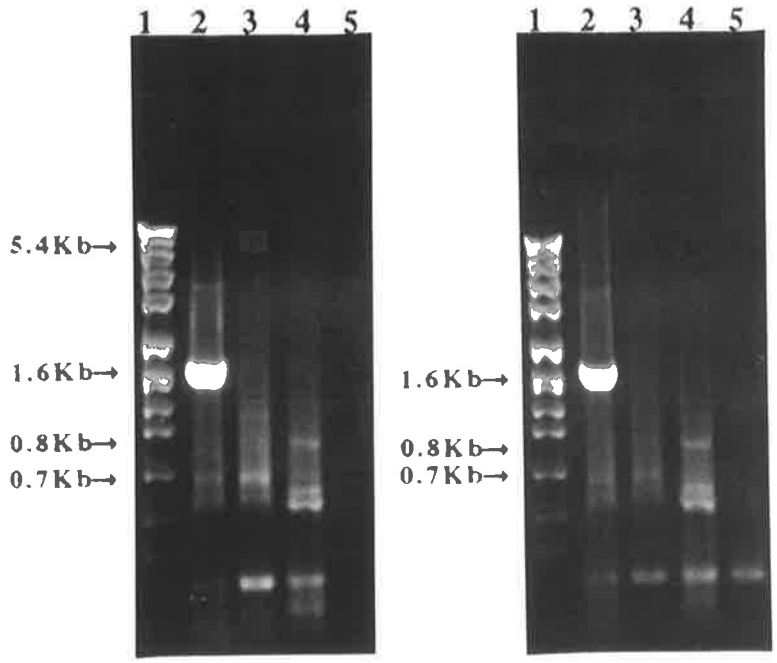


PCR analysis by the final primer pair L567-H1452, resulted in the amplification of 4 distinct DNA bands which had sizes of approximately 1.0Kb, 0.8Kb, 0.7Kb and 0.6Kb (Graph 4.1.1D). These products were generated from samples within each age group. Again, the percentage of 10 week old mtDNA samples which resulted in the synthesis of these DNA products (70%, 60%, 60%, and 60% respectively), was greater than or approximately equal to the corresponding percentages of the other older age groups.

4.1.2. Analysis of Heart mtDNA by part A of the developed PCR method:

Representative gels showing the results obtained when a 10 week old and a 42 month old mouse heart mtDNA sample were analyzed by part A of the developed PCR method, are shown in Figures 4.1.2A and 4.1.2B respectively. These two heart mtDNA samples were analyzed on the same day using identical reaction conditions. Within lane 2 of each figure, a distinct 1.6Kb band was observed, which corresponds to the expected L567-H732 PCR product. In addition, two very faint bands of 0.6Kb and 0.7Kb were also observed within this same lane of each figure. With the primer pair L567-H1107, a very faint band of 5.4Kb, corresponding to the expected PCR product, was generated from the 10 week old sample (Figure 4.1.2A, lane 3), along with two smaller products of approximately 0.6Kb and 0.7Kb. Analysis of the 42 month old mtDNA sample by this primer pair resulted in only a faint 0.7Kb band being observed as shown in lane 3 of the Figure 4.1.2B.

Three distinct products of 0.8Kb, 0.65Kb and 0.55Kb were synthesized from both of these samples by the primers L567 and H1295 (lane 4 in each figure). Shifting the H primer to H1452, resulted in no products being amplified from either of these two heart mtDNA samples (lane 5 of each figure). A small band of approximately 100bp was observed within this lane for the 42 month old sample however, this size fragment was commonly synthesized in all the PCR reactions regardless of the primer pair combination, as can be seen in the other previous lanes within these figures.



Figures 4.1.2A & B. Analysis of Heart mtDNA by Part A of the developed PCR method:
Heart mtDNA from a 10 week old and a 42 month old mouse were analyzed by part A of the previously developed PCR method (Figures 4.1.2A & B respectively). This involved the analysis of each sample by the following primer pair combinations; L567-H732, L567-H1107, L567-H1295 and L567-H1452 (lanes 2, 3, 4 and 5 respectively within each figure). DNA size markers ranging from 8.5Kb to 0.36Kb were run in lane 1 of each figure.

Reviewing the part A PCR analysis of both of these heart mtDNA samples, the only band which displayed a H strand primer shift pattern was the expected, L567-H732 PCR product of 1.6Kb generated from the 10 week old mtDNA sample (Figure 4.1.2A, lane 2), which showed a corresponding increase in size to 5.4Kb when the H strand primer was shifted to the nucleotide position 11072 (lane 3).

As previously mentioned, these figures only represent the analysis of one heart mtDNA sample from each age group, and the banding pattern observed for each sample varied slightly. A summary of the products generated from all the heart mtDNA samples within each age group is displayed in the Graphs 4.1.2A-D. In the Graph 4.1.2A, which corresponds to the results obtained with the primer pair L567-H732, the number of heart mtDNA samples which resulted in the amplification of a particular size DNA product is expressed as a percentage of the total number of DNA samples examined within that age group. As can be seen, 100% of all the heart mtDNA samples examined from both age groups, resulted in the synthesis of the expected 1.6Kb PCR product. The percentage of 10 week old mtDNA samples which generated the two smaller products of 0.7Kb and 0.6Kb were 20% and 50% respectively, which were both greater than those of the older 42 month old mtDNA samples.

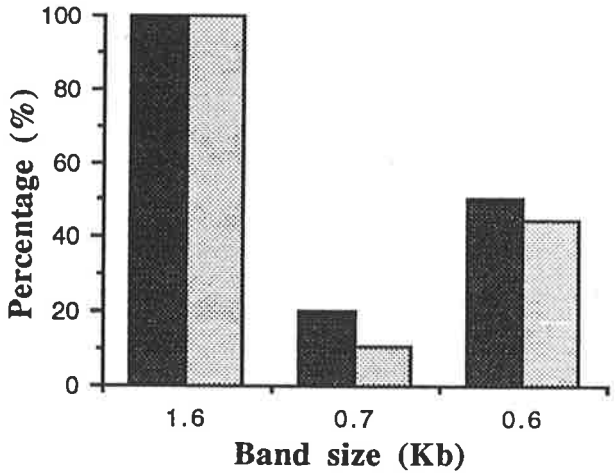
Analysis by the primer pair L567-H1107, resulted in the generation of 5 different products with sizes of 5.4Kb, 1.0Kb, 0.8Kb, 0.7Kb and 0.6Kb (Graph 4.1.2B). All of these bands were observed following the analysis of both young and old heart mtDNA samples. The percentage of 10 week old mtDNA samples which generated each of these products (30%, 20%, 20%, 40% and 30% respectively), was greater than or approximately equal to the percentage of 42 month old samples which also generated these bands.

Four DNA products of 1.0Kb, 0.8Kb, 0.65Kb and 0.55Kb were synthesized by the primers L567 and H1295, as shown in the Graph 4.1.2C. All of these products were generated from samples within each age group. The percentage of 10 week old mtDNA samples which resulted in the synthesis of the 1.0Kb and the 0.65Kb products were 30% and 70% respectively, which were greater than the percentage of 42

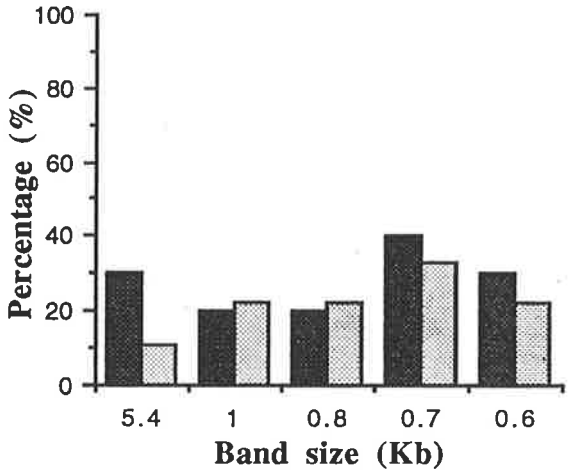
Graphs 4.1.2A, B, C & D. Analysis of Heart mtDNA by part A of the developed PCR method:

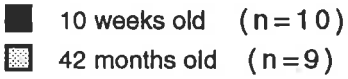
These graphs display the size (kilobases) of the DNA products amplified from 10 week old and a 42 month old mouse heart mtDNA samples, by each primer pair combination of part A of the developed PCR method. This analysis involved the following pairs; L567-H732, L567-H1107, L567-H1295 and L567-H1452, with the results shown in the graphs 4.1.2A, B, C & D respectively. The number of heart mtDNA samples which resulted in the amplification of a particular product is expressed as a % of the total number of DNA samples examined within that age group.

Graph 4.1.2A. L567-H732

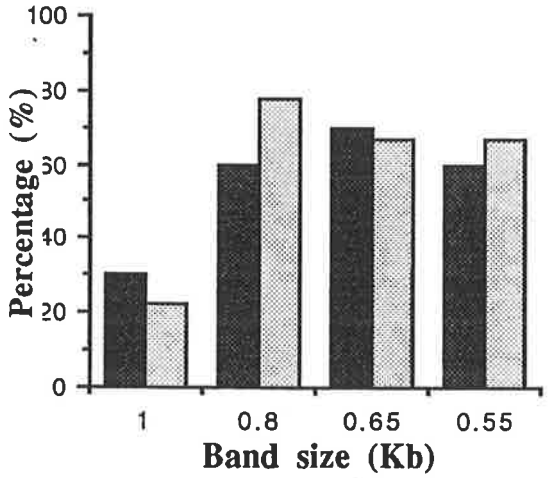


Graph 4.1.2B. L567-H1107

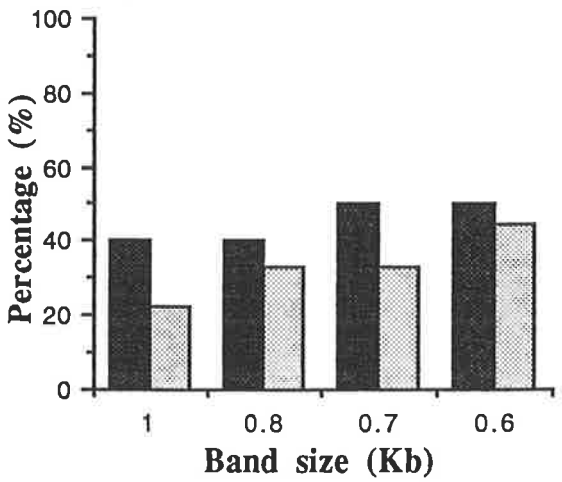




Graph 4.1.2C. L567-H1295



Graph 4.1.2D. L567-H1452



month old samples. However, the percentage of 42 month old samples which resulted in the formation of the 0.8Kb and the 0.55Kb products was 77.7% and 66.6% respectively, which were higher than the corresponding percentages of 10 week old samples which synthesized these DNA fragments.

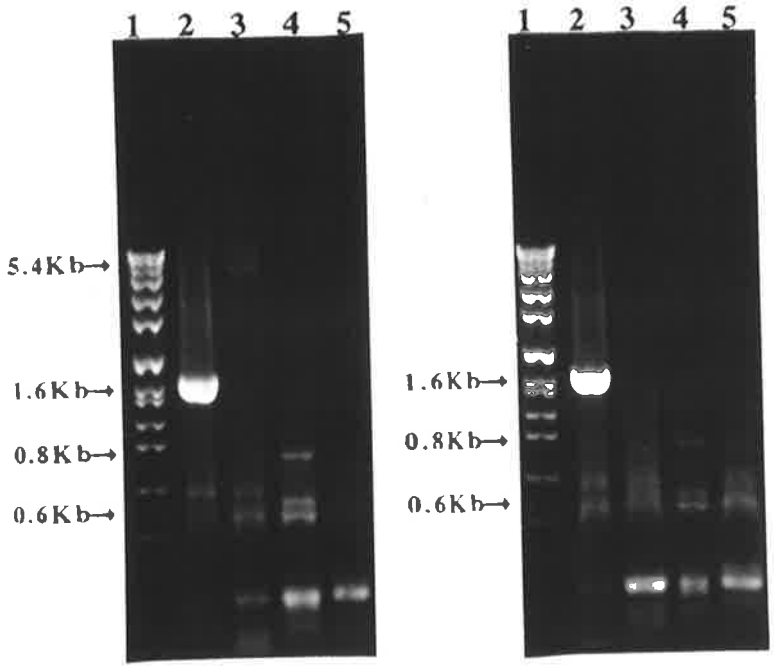
PCR analysis with the final primer pair L567-H1452, resulted in the generation of four different products of approximately 1.0Kb, 0.8Kb, 0.7Kb and 0.6Kb (Graph 4.1.2D). All of these products were synthesized from both 10 week old and 42 month old mtDNA samples. The percentage of 10 week old samples which resulted in these products being amplified (40%, 40%, 50% and 50% respectively) was greater than the percentage of 42 month old samples which also produced these products.

4.1.3. Analysis of skeletal muscle mtDNA by part A of the developed PCR method:

Representative gels showing the results obtained when a 10 week old and a 42 month old muscle mtDNA sample, were analyzed by part A of the developed PCR method, are shown in Figures 4.1.3A and 4.1.3B respectively. These two muscle mtDNA samples were analyzed on the same day using the same reaction conditions. Within lane 2 of each figure, a distinct 1.6Kb band was detected, which corresponds to the expected L567-H732 PCR product. In addition, two smaller products of 0.7Kb and 0.6Kb were also detected within both of these gels.

PCR analysis of the 10 week old mtDNA sample with the primer pair L567-H1107 resulted in the generation of two distinct products of approximately 0.6Kb and 0.7Kb, and a larger very faint band of 5.4Kb, which corresponds to the expected PCR product (Figure 4.1.3A, lane 3). Analysis of the 42 month old sample by these same primers resulted in two faint smeared bands of 0.6Kb and 0.7Kb being observed (Figure 4.1.3B, lane 3).

Three distinct products of 0.8Kb, 0.65Kb and 0.55Kb were generated from both the young and the old muscle mtDNA sample by the primer pair L567-H1295 (lane 4 in each figure). Shifting the H strand primer to H1452 resulted in two smeared



4.1.3A 4.1.3B

Figures 4.1.3A & B. Analysis of Muscle mtDNA by part A of the developed PCR method:
Skeletal muscle mtDNA from a 10 week old and a 42 month old mouse were analyzed by part A of the previously developed PCR method (Figures 4.1.3A & B respectively). This involved the analysis of each sample by the following primer pair combinations; L567-H732, L567-H1107, L567-H1295 and L567-H1452 (lanes 2, 3, 4 and 5 respectively within each figure). DNA size markers ranging from 8.5Kb to 0.36Kb were run in lane 1 of each figure.

bands of approximately 0.6Kb and 0.7Kb being observed following the analysis of the 42 month old sample (Figure 4.1.3B, lane 5), while the only product generated by these primers from the 10 week old sample was a 100bp band (Figure 4.1.3A, lane 5), which is typically generated in virtually all of the PCR reactions regardless of the primer pair combination, as can be seen in the other lanes of both of these figures.

Therefore, reviewing all the products generated from the PCR analysis of this young and old muscle mtDNA sample, the only band which showed a primer shift pattern was the 1.6Kb product generated during the analysis of the 10 week old sample by the primers L567 and H732 (Figure 4.1.3A, lane 2). This expected PCR product of 1.6Kb displayed a corresponding increase in size to 5.4Kb when the H strand primer was changed to the nucleotide position 11072 (lane 3).

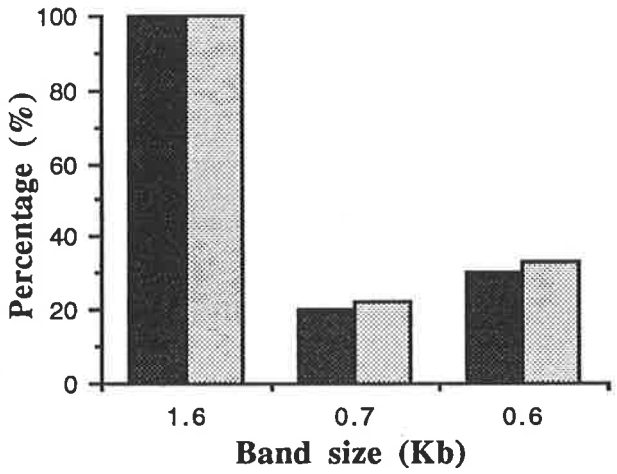
As previously stated, these figures only represent the results obtained from the analysis of one muscle mtDNA sample from each age group, and the banding pattern observed for each sample varied. A summary of the results obtained from the analysis of all the samples within each age group can be seen in the Graphs 4.1.3A-D. In Graph 4.1.3A, which corresponds to the results obtained with the primer pair L567-H732, the number of muscle mtDNA samples which resulted in the amplification of a particular size product is expressed as a percentage of the total number of DNA samples examined within that age group. As can be seen, 100% of all the muscle mtDNA samples examined within each age group, displayed the expected 1.6Kb product. In addition, two smaller products of 0.7Kb and 0.6Kb were also observed with equal percentages of the young and the old mtDNA samples generating each of these products.

PCR analysis with the primers L567 and H1107, resulted in a total of five DNA products being synthesized with approximate sizes of 5.4Kb, 1.0Kb, 0.8Kb, 0.7Kb and 0.6Kb (Graph 4.1.3B). All of these products were generated from both young and the old mtDNA samples analyzed. The percentage of 10 week old muscle mtDNA samples which resulted in the generation of the expected PCR product of 5.4Kb and the other bands of 1.0Kb, 0.8Kb, 0.7Kb and 0.6Kb (30%, 10%, 20%, 40% and 50% respectively), were greater than or approximately equal to the percentage of the 42

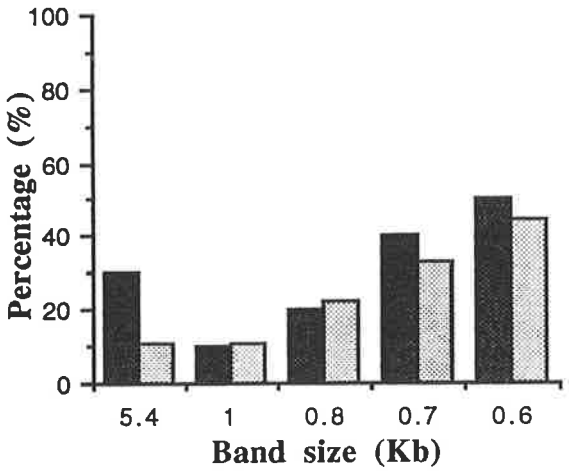
Graphs 4.1.3A, B, C & D. Analysis of Muscle mtDNA by part A of the developed PCR method:

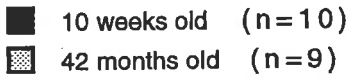
These graphs display the size (kilobases) of the DNA products generated from 10 week old and 42 month old mouse muscle mtDNA samples, by each primer pair combination of part A of the developed PCR method. This analysis involved the following pairs; L567-H732, L567-H1107, L567-H1295 and L567-H1452, with the results shown in the graphs 4.1.3A, B, C & D respectively. The number of muscle mtDNA samples which resulted in the amplification of a particular band is expressed as a % of the total number of DNA samples examined within that age group.

Graph 4.1.3A. L567-H732

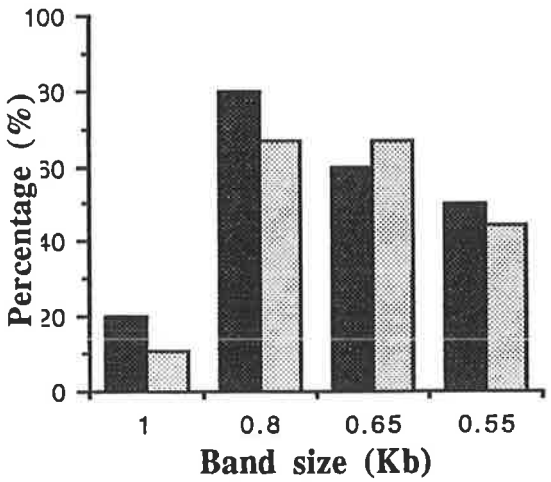


Graph 4.1.3B. L567-H1107

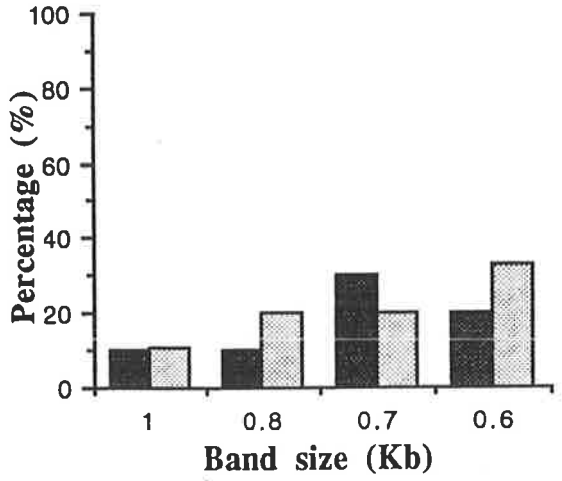




Graph 4.1.3C. L567-H1295



Graph 4.1.3D. L567-H1452



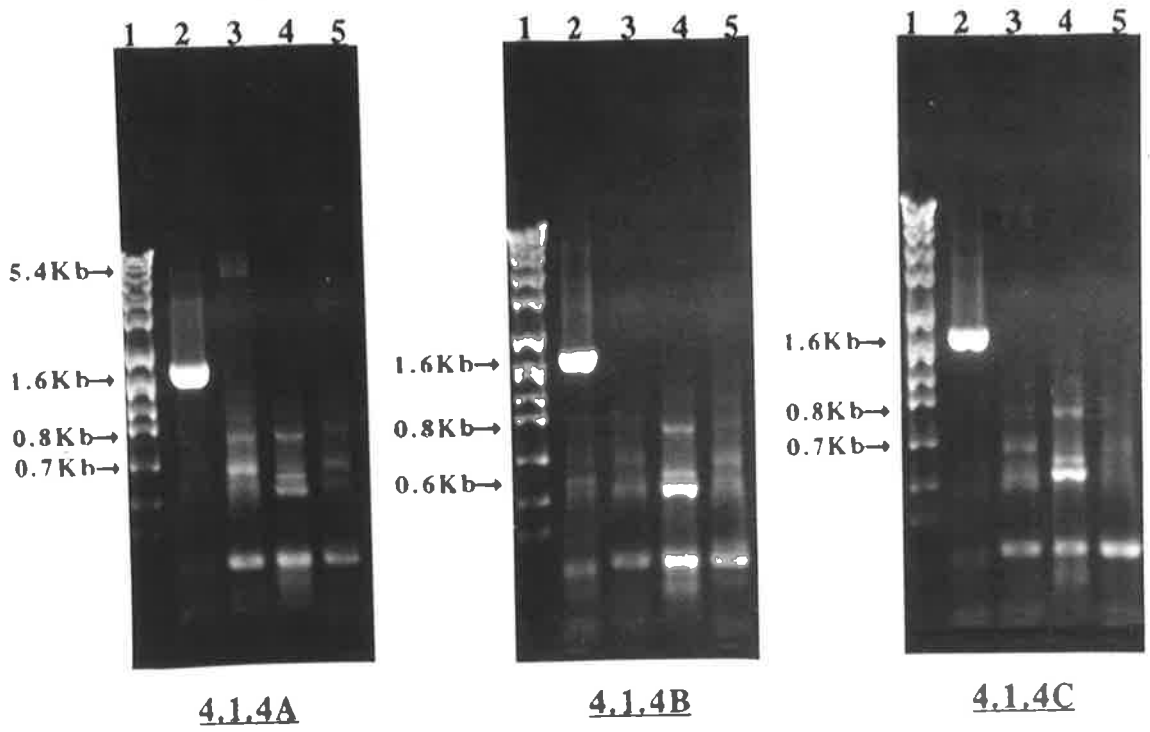
month old samples which also generated these products.

Four products of approximately 1.0Kb, 0.8Kb, 0.65Kb and 0.55Kb were generated when samples from each of these age groups were analyzed by the primer pair L567-H1295 (Graph 4.1.3C). The percentage of 10 week old samples which generated these bands (20%, 80%, 60% and 50 % respectively), was slightly greater than or approximately equal to that of the 42 month old samples. Shifting the H primer to H1452 resulted in the synthesis of four products being amplified from samples within each age group (Graph 4.1.3D). A band of 0.7Kb was detected in 30% and 20% of the young and old mtDNA samples respectively, while the percentage of 10 week old samples which generated the other products (1.0Kb, 0.8Kb and 0.6Kb), was slightly lower than or approximately equal to the corresponding percentages of the 42 month old samples.

4.1.4. Analysis of liver mtDNA by part A of the developed PCR method:

Gels representing the part A PCR analysis of a 10 week old, a 19 month old and a 42 month old liver mtDNA sample, are shown in Figures 4.1.4A-C respectively. These three samples were analyzed on the same day using the same reaction conditions. Analysis by the primer pair L567-H732, resulted in the expected PCR product of 1.6Kb being generated from each mtDNA sample examined (lane 2 of each figure). In addition, a smaller band of approximately 0.6Kb was also detected within this lane from the 19 month old sample (Figure 4.1.4B, lane 2). With the primers L567 and H1107, a faint band corresponding to the expected PCR product of 5.4Kb was generated from the 10 week old sample (Figure 4.1.4A, lane 3), along with two smaller products of 0.8Kb and 0.7Kb. Analysis of the 19 month old and the 42 month samples by these same primers resulted in the formation of two products of 0.6Kb and 0.7Kb being observed (Figure 4.1.4B & C, lane 3).

Three distinct products of 0.8Kb, 0.65Kb and 0.55Kb were observed following the L567-H1295 PCR analysis of each of the different aged liver mtDNA



Figures 4.1.4A, B & C. Analysis of Liver mtDNA by part A of the developed PCR method:
Liver mtDNA from a 10 week old, a 19 month old and a 42 month old mouse were analyzed by part A of the previously developed PCR method (Figures 4.1.4A, B & C respectively). This involved the analysis of each sample by the following primer pair combinations; L567-H732, L567-H1107, L567-H1295 and L567-H1452 (lanes 2, 3, 4 and 5 respectively within each figure). DNA size markers ranging from 8.5Kb to 0.36Kb were run in lane 1 of each figure.

samples (lane 4 of each figure). Shifting the H strand primer to H1452, resulted in a smearing pattern being observed for the 42 month old sample (Figure 4.1.4C, lane 5), while the 19 month old sample generated 2 faint smeared bands of 0.7Kb and 0.6Kb (Figure 4.1.4B, lane 5), and the 10 week old mtDNA sample generated 2 distinct products of approximately 0.8Kb and 0.7Kb (Figure 4.1.4A, lane 5).

Reviewing all the bands generated within each of these 3 figures, the only band which displayed a H strand primer shift pattern was the 1.6Kb product generated from the 10 week old sample by the primers L567 and H732 (Figure 4.1.4A, lane 2). This expected PCR product displayed a corresponding increase in size when the H primer was changed to H1107 (lane 3).

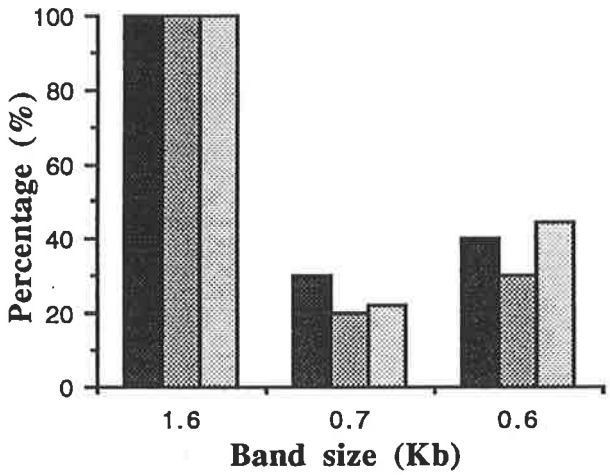
As previously stated, these figures only represent one liver mtDNA sample from each age group, and the banding pattern observed for each liver mtDNA sample varied. A summary of the results obtained from the analysis of all the samples within each age group can be seen in the Graphs 4.1.4A-D. In the Graph 4.1.4A, which corresponds to the results obtained with the primer pair L567-H732, the number of liver mtDNA samples which resulted in the amplification of a particular size product is expressed as a percentage of the total number of DNA samples examined within that age group. As can be seen, 100% of all the liver mtDNA samples examined within each age group, displayed the expected 1.6Kb product. In addition, two smaller bands of 0.7Kb and 0.6Kb were also observed, with the percentage of 10 week old, 19 month old, and 42 month old samples generating the 0.7Kb product being 30%, 20% and 22.2% respectively, while the % of samples which resulted in the 0.6Kb fragment being amplified were 40%, 30% and 44.4% respectively.

PCR analysis with the primers L567 and H1107 resulted in 5 products being amplified with sizes of 5.4Kb, 1.0Kb, 0.8Kb, 0.7Kb and 0.6Kb (Graph 4.1.4B). All of these products were generated from samples within each age group. The expected 5.4Kb product was amplified from 20% of the young samples, whilst being synthesized from 10% and 33.3% of the older mtDNA samples respectively. The percentage of 10 week old liver mtDNA samples which resulted in the generation

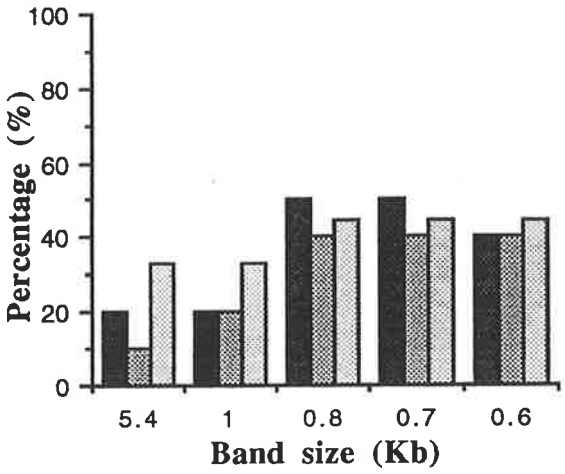
Graphs 4.1.4A, B, C & D. Analysis of Liver mtDNA by part A of the developed PCR method:

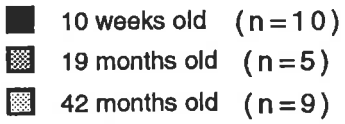
These graphs display the size (kilobases) of the products generated from various aged mouse liver mtDNA samples (10 weeks old, 19 months old and 42 months old), by each primer pair combination of part A of the developed PCR method. This analysis involved the following pairs; L567-H732, L567-H1107, L567-H1295 and L567-H1452, with the results shown in the graphs 4.1.4A, B, C & D respectively. The number of liver mtDNA samples which resulted in the amplification of a particular product is expressed as a % of the total number of DNA samples examined within that age group.

Graph 4.1.4A. L567-H732

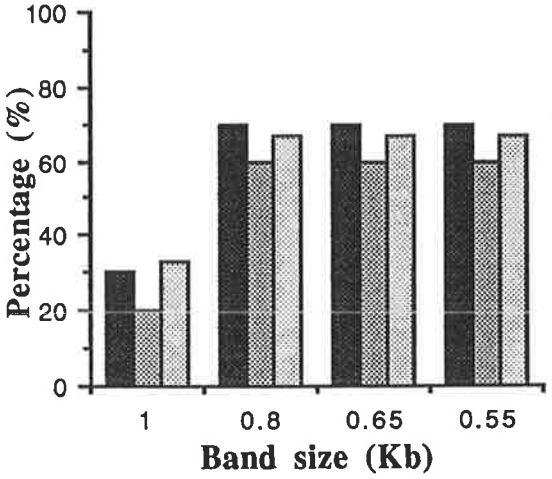


Graph 4.1.4B. L567-H1107

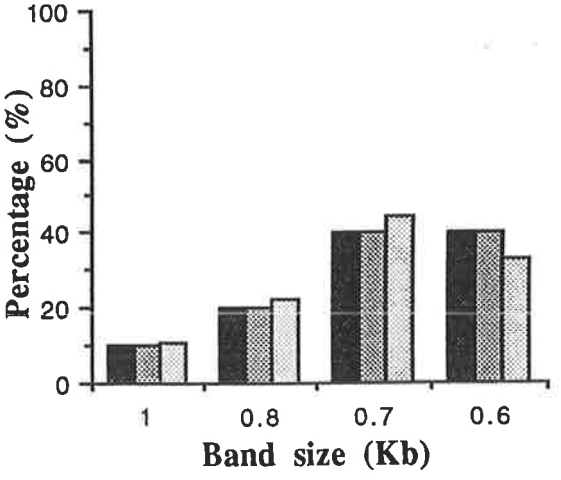




Graph 4.1.4C. L567-H1295



Graph 4.1.4D. L567-H1452



of the 0.8Kb and 0.7Kb products was greater than that of the older samples, while the % of 42 month old samples which produced the 1.0Kb and 0.6Kb products were greater than those of the 10 week and 19 month old samples.

Four distinct products of 1.0Kb, 0.8Kb, 0.65Kb and 0.55Kb were amplified by the primer pair L567-H1295 from samples within each age group (Graph 4.1.4C). The percentage of 10 week old samples which resulted in these products being synthesized (30%, 70%, 70% and 70% respectively), was greater than or approximately equal to the percentage of 19 month and 42 month old samples which also generated these bands when analyzed by the same primer pair. Subsequently changing the H primer to H1452 resulted in four products of approximately 1.0Kb, 0.8Kb, 0.7Kb and 0.6Kb being generated from DNA samples within each age group (Graph 4.1.4D). Again, the percentage of 10 week old samples which resulted in the synthesis of these products (10%, 20%, 40% and 40% respectively), was greater than or approximately equal to the percentage of 19 month and 42 month old mtDNA samples which also displayed these bands when analyzed by the same primers.

Summary:-

MtDNA isolated from four different tissues was analyzed by part A of the previously developed PCR method for detecting deleted DNA molecules. Although the banding pattern observed varied slightly between the different samples, overall the size and number of products generated by each primer pair combination was the same regardless of the age of the animal from which the sample was obtained. The percentage of mtDNA samples obtained from 10 week old mice which generated these products, was in general greater than or equal to the percentage of samples obtained from the older animals which also synthesized these products. This suggests that if any of these products were derived from deleted mtDNA templates, they were not age-related deletions.

The only PCR product which displayed a H strand primer shift pattern was the 1.6Kb PCR product synthesized from all the samples by the primer pair L567-H732. This expected PCR product showed a corresponding increase in size to 5.4Kb when the H strand primer was shifted to the nucleotide position 11072. However, none of the other smaller products displayed this H strand primer shift pattern which suggests that these products are not due to the presence of deleted mtDNA molecules, but are rather artifacts of the PCR technique, such as non-specific products generated by the PCR primers misannealing to the wildtype mtDNA template.

Finally, comparing the results obtained for each tissue, the same size and number of products were synthesized irrespective of the tissue examined, implying that none of the products were tissue specific.

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

Experimental design:-

Various aged brain mtDNA samples (10 weeks old, 19 months old and 42 months old), were analyzed by part B of the developed two-part PCR method, to confirm if any of the bands previously generated by part A were deleted PCR products, by showing if they were capable of displaying an L strand and/or a H strand primer shift pattern. This second PCR analysis involved 8 PCR reactions which contained the following primer pair combinations; L567-H732, L480-H732, L567-H1107, L480-H1107, L567-H1295, L480-H1295, L567-H1452 and L480-H1452. Only the brain mtDNA samples were analyzed by part B because, the brain is the most likely non-replicative tissue to contain deleted mtDNA molecules and three different age groups of

brain mtDNA (10 weeks old, 19 months old and 42 months old) were available for analysis. Also, since each of the four tissues previously examined by part A generated the same size and number of smaller than expected PCR product bands, if any of these bands represent deleted PCR products this could be confirmed by analyzing any one of these tissues.

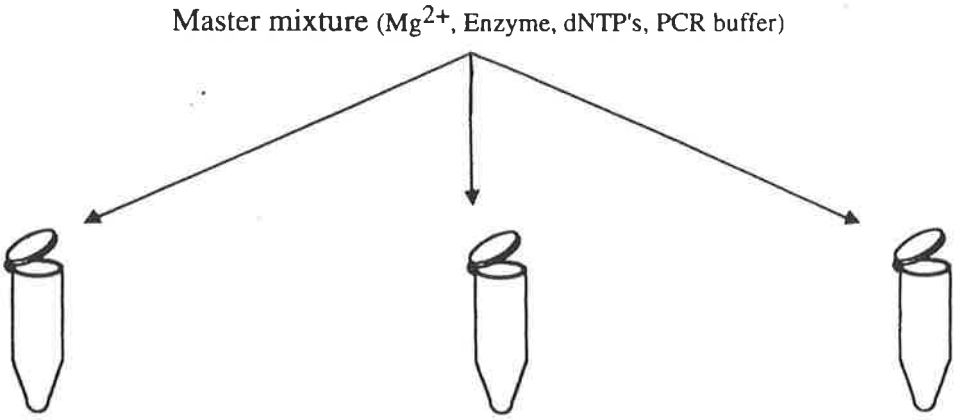
Within any experiment, at least one brain mtDNA sample from each of the 3 age groups was examined at the same time. This involved preparing a master mixture of all the PCR reaction reagents (omitting the template DNA and the primers), and aliquoting this into 3 separate reaction tubes (see Diagram 4.2). The desired DNA template corresponding to each age group was then added to one of each of these 3 tubes. The mixture within each tube was then aliquoted into 8 separate reaction tubes, where each contained a different primer pair combination of part B of the developed PCR method. All of the reaction tubes were then placed within the DNA thermal cycling machine together, and aliquots of each reaction were subsequently loaded onto the same agarose gel and stained with ethidium bromide.

Within each age group, the sizes of the products generated by each primer pair combination were calculated, and examined to determine if any particular band displayed a corresponding shift in size when either the position of the H or the L strand primers were shifted. The results obtained from each age group were then compared to determine if the amplification of any of these products was age dependent.

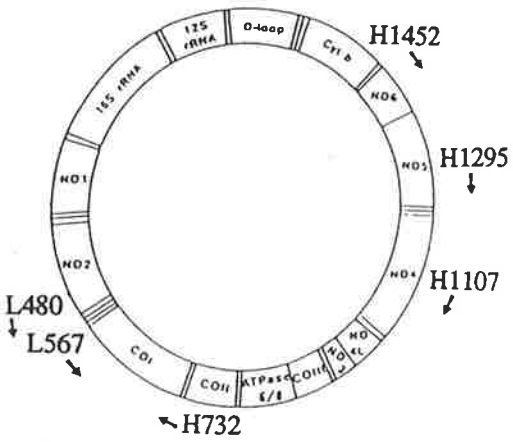
Results:-

Representative gels showing the results obtained when a 10 week old, a 19 month old and a 42 month old brain mtDNA sample were analyzed by part B of the developed PCR method, are shown in Figures 4.2A-C respectively. These DNA samples were analyzed on the same day using identical reaction conditions. The expected PCR product of 1.6Kb, generated by the primer pair L567-H732, was

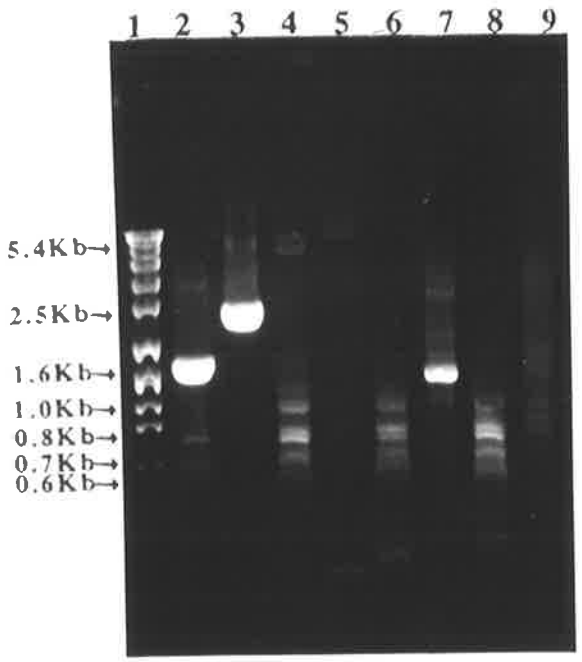
Diagram 4.2. Experimental protocol for analyzing mice brain mtDNA by part B of the developed PCR method:



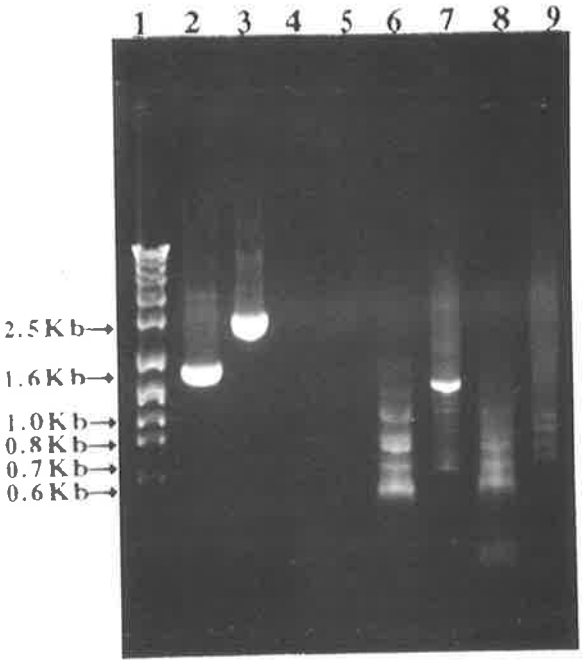
+ 10 week old mtDNA	+19 month old mtDNA	+ 42 month old mtDNA
1. L567-H732	1. L567-H732	1. L567-H732
2. L480-H732	2. L480-H732	2. L480-H732
3. L567-H1107	3. L567-H1107	3. L567-H1107
4. L480-H1107	4. L480-H1107	4. L480-H1107
5. L567-H1295	5. L567-H1295	5. L567-H1295
6. L480-H1295	6. L480-H1295	6. L480-H1295
7. L567-H1452	7. L567-H1452	7. L567-H1452
8. L480-H1452	8. L480-H1452	8. L480-H1452



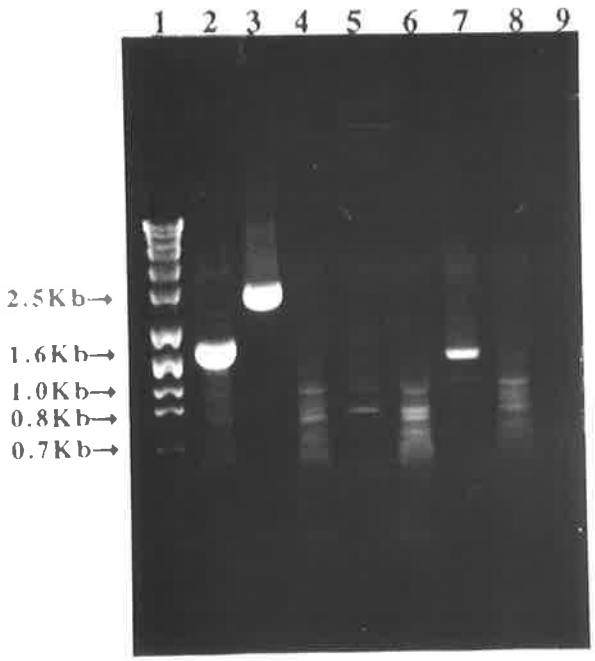
All 24 tubes were placed within the thermal cycling machine together, and an aliquot of each reaction was subsequently run on an agarose gel.



4.2A



4.2B



4.2C

Figures 4.2A, B & C. Analysis of Brain mtDNA by part B of the developed PCR method:
Brain mtDNA from a 10 week old, a 19 month old and a 42 month old mouse were analyzed by part B of the previously developed PCR method (Figures 4.2A, B and C respectively). This involved the analysis of each sample by the following primer pair combinations; L567-H732, L480-H732, L567-H1107, L480-H1107, L567-H1295, L480-H1295, L567-H1452 and L480-H1452 (lanes 2, 3, 4, 5, 6, 7, 8 and 9 respectively within each figure). DNA size markers ranging from 8.5Kb to 0.36Kb were run in lane 1 of each figure.

detected following the analysis of all 3 DNA samples, as shown in lane 2 of each figure. In addition, a smaller band of approximately 0.8Kb was also detected within this lane for the 10 week old sample (Figure 4.2A). By changing the L primer to L480 (lane 3 of each figure), a definite product of 2.5Kb was observed following the analysis of each DNA sample, which corresponds to the expected PCR product generated by this primer pair L480-H732.

PCR analysis with the primers L567 and H1107 resulted in 4 products being amplified from the 10 week old sample with sizes of 1.0Kb, 0.8Kb, 0.7Kb and 5.4Kb which corresponds to the expected PCR product (Figure 4.2A, lane 4). Analysis of the 19 month old brain mtDNA sample by these same primers did not generate any products (Figure 4.2B, lane 4), however, analysis of the 42 month old sample resulted in the amplification of two DNA bands of 1.0Kb and 0.8Kb (Figure 4.2C, lane 4). Shifting the L primer to L480, resulted in a product of approximately 0.85Kb being generated during the analysis of the 42 month old sample (Figure 4.2C, lane 5), while an extremely faint band of 6.3Kb was observed following the analysis of the 10 week old sample, which corresponds to the expected PCR product (Figure 4.2A, lane 5). No products were generated from the 19 month old sample with this primer pair combination (Figure 4.2B, lane 5).

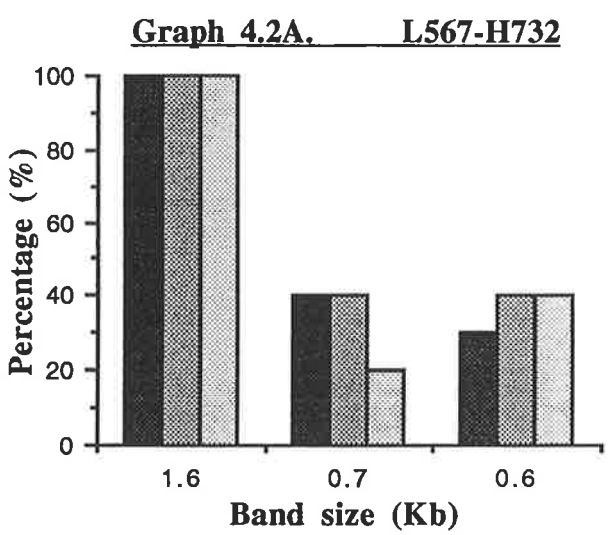
With the primer pair L567-H1295 (lane 6 of each figure), examination of both the 10 week old and the 42 month DNA samples resulted in the synthesis of two products of 1.0Kb and 0.8Kb, with a smeared pattern extending from 0.7-0.5Kb also being observed (Figures 4.2A & C, lane 6). Analysis of the 19 month old sample by these same primers resulted in the amplification of four products of 1.0Kb, 0.8Kb, 0.7Kb and 0.55Kb (Figure 4.2B, lane 6). Shifting the L strand primer to L480 resulted in a definite product of approximately 1.3Kb being synthesized from each DNA sample, along with a smaller band of approximately 1.1Kb also being observed (lane 7 of each figure). In addition, both the 10 week old, and the 42 month old samples also generated a faint band of 3.0Kb (Figures 4.2A & C), while the 19 month old sample produced an additional product of 0.7Kb (Figure 4.2B).

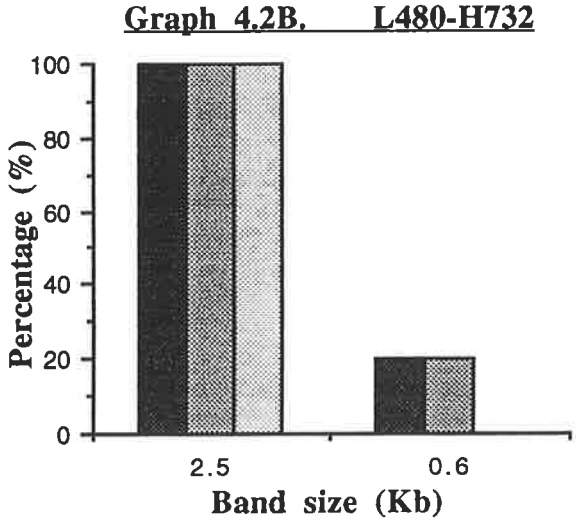
The primers L567 and H1452 generated four products during the analysis of both the 10 week old and the 19 month old samples (Figures 4.2A & B, lane 8). These products had sizes of approximately, 1.0Kb, 0.8Kb, 0.7Kb and 0.6Kb, while two bands of 1.0Kb and 0.8Kb were detected against a streaked background pattern for the 42 month old sample (gel 4.2C, lane 8). Changing the L strand primer to L480, resulted in two very faint bands of 1.0Kb and 0.9Kb being observed following the analysis of the 10 week old and the 19 month old DNA samples (Figures 4.2A & B, lane 9), while no products were visible following the analysis of the oldest, 42 month old sample (Figure 4.2C, lane 9).

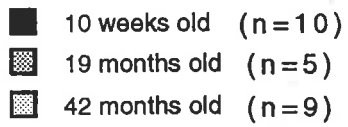
As previously mentioned, these 3 figures only represent one brain mtDNA sample from each of the 3 different age groups, and the banding pattern observed for each sample varied slightly. A summary of the results obtained from all the samples within each age group can be seen in the Graphs 4.2A-H, which represent each of the different primer pair combinations. In the Graph 4.2A, which corresponds to the PCR analysis by the primer pair L567-H732, the number of brain mtDNA samples which resulted in the amplification of a particular size product is expressed as a percentage of the total number of DNA samples examined within that age group. From this graph it can be seen that 100% of all the mtDNA samples examined within each age group, resulted in the expected 1.6Kb product being synthesized. In addition, a smaller product of 0.7Kb was generated from 40, 40 and 20% of the 10 week old, 19 month old and the 42 month old samples respectively, while a 0.6Kb product was amplified from 30, 40 and 40% of the DNA samples respectively.

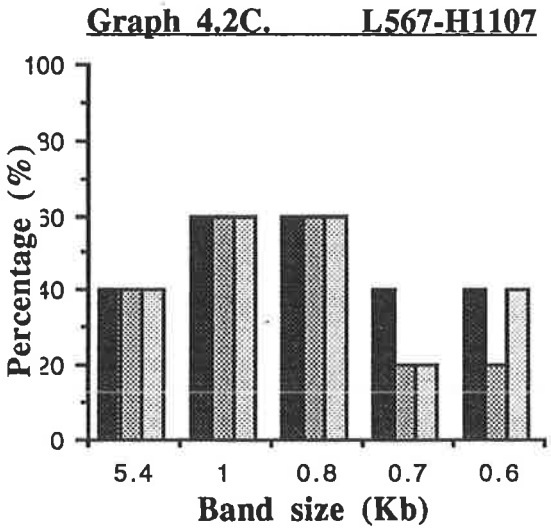
Only the 1.6Kb fragment observed in this lane displayed a corresponding increase in size when the L primer was changed to L480, resulting in a 2.5Kb product being detected (Graph 4.2B). This expected PCR product of 2.5Kb was generated from 100% of all the samples examined within each age group. In addition, a smaller product of 0.6Kb was also synthesized by these primers L480-H732, with the percentage of 10 week old samples generating this product being equal to or greater than the percentage of the other two older age groups.

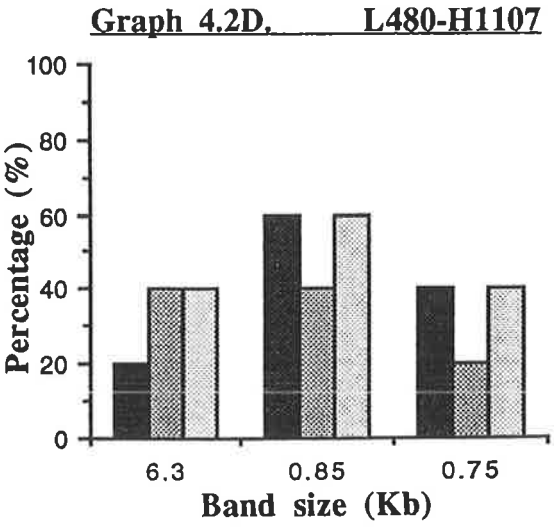
Graphs 4.2 A-H. Analysis of Brain mtDNA by part B of the developed PCR method:
These graphs display the size (kilobases) of the products generated from various aged mouse brain mtDNA samples (10 weeks old, 19 months old and 42 months old), by each primer pair combination of part B of the developed PCR method. This analysis involved the following primer pairs; L567-H732, L480-H732, L567-H1107, L480-H1107, L567-H1295, L480-H1295, L567-H1452 and L480-H1452, with the results shown in the graphs 4.2A, B, C, D, E, F, G & H respectively. The number of brain mtDNA samples which resulted in the amplification of a particular product is expressed as a % of the total number of DNA samples examined within that age group.





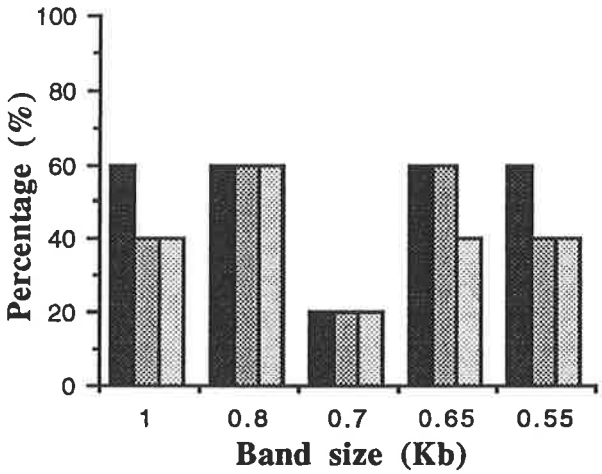




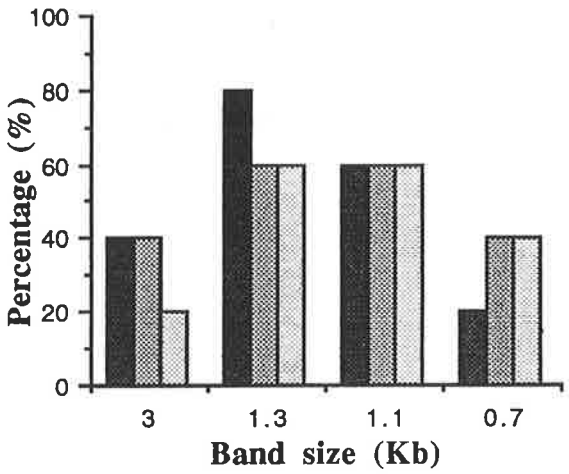


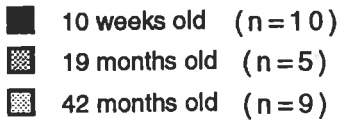
Graphs 4.2A-H. (continued) Analysis of Brain mtDNA by part B of the developed PCR method:

Graph 4.2E. L567-H1295

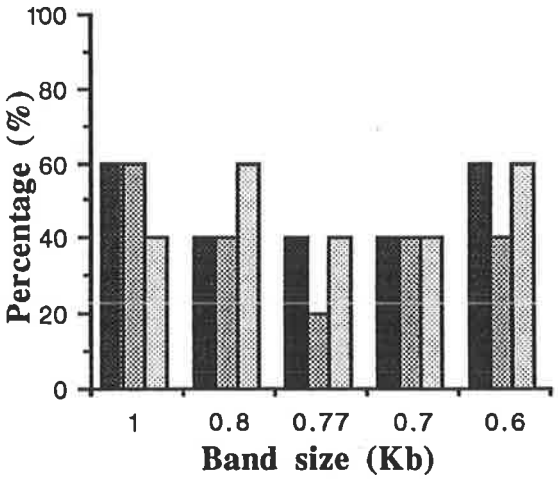


Graph 4.2F. L480-H1295

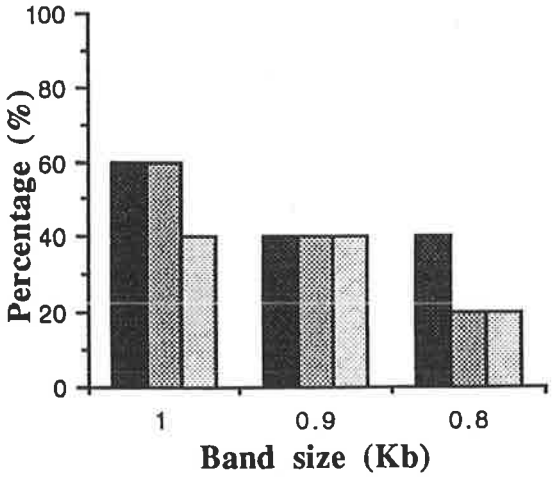




Graph 4.2G. L567-H1452



Graph 4.2H. L480-H1452



Analysis by the primers L567 and H1107, resulted in 5 products being amplified with sizes of 5.4Kb, 1.0Kb, 0.8Kb, 0.7Kb and 0.6Kb (Graph 4.2C). The expected 5.4Kb PCR product was generated from 40% of the samples within each age group, while the percentage of 10 week old samples which resulted in the other products being amplified was greater than or equal to the percentage of the older mtDNA samples. The 5.4Kb product was the only band which displayed a corresponding increase in size when the L primer was changed to L480, generating a product of 6.3Kb (Graph 4.2D). This expected PCR product of 6.3Kb was generated from 20%, 40% and 40% of the 10 week old, 19 month and 42 month old samples respectively. In addition, this primer pair L480-H1107 also generated two products of 0.85Kb and 0.75Kb, and the percentage of 10 week old samples which resulted in the synthesis of these products was greater than or equal to the corresponding percentages of the older two age groups.

PCR analysis with the primers L567 and H1295, resulted in a total of 5 products being amplified with sizes of 1.0Kb, 0.8Kb, 0.7Kb, 0.65Kb and 0.55Kb (Graph 4.2E). The percentage of 10 week old samples which resulted in the synthesis of each of these bands (60, 60, 20, 60 and 60% respectively), was greater than or equal to the percentage calculated for the older mtDNA samples. None of these products showed a definite corresponding increase in size when the L primer was shift to L480 however, a band of 1.3Kb was observed (Graph 4.2F), which is similar in size to the expected 1.42Kb product which would be generated if the previous 0.55Kb band represented a deleted PCR product (Graph 4.2E). However, this 1.3Kb product was amplified from samples within each age group where 80% of the 10 week old samples resulted in this product being synthesized, which is greater than the percentage of samples from the older aged groups. Also, the previously observed 0.55Kb product (Graph 4.2E) failed to display a H strand primer shift pattern. Thus, this would imply that this product does not represent an age-related deleted PCR product.

In addition to this 1.3Kb band, the primers L480 and H1295 also generated 3 other products of 3.0Kb, 1.1Kb and 0.7Kb (Graph 4.2F). The percentage of 10 week

old samples which generated these productss was greater than or equal to the corresponding percentages of the older DNA samples, except for the 0.7Kb product which was generated during the analysis of 20, 40 and 40% of the 10 week old, 19 month old and the 42 month old samples respectively.

The primer pair L567-H1452, resulted in a total of 4 products being amplified with sizes of 1.0Kb, 0.8Kb, 0.7Kb and 0.6Kb (Graph 4.2G). The percentage of 10 week old samples which displayed these bands was greater than the respective percentages of the older DNA samples, except for the 0.8Kb product which was only generated from 40% of both the 10 week old and the 19 month old samples, whereas it was produced from 60% of the 42 month old samples. Shifting the L primer to L480 did not result in a corresponding increase in size of any of these bands. This primer pair combination of L480-H1452, resulted in the synthesis of 3 products of approximately 1.0Kb, 0.9Kb and 0.8Kb (Graph 4.2H). The percentage of 10 week old mtDNA samples which resulted in the production of each of these bands was equal to or greater than the corresponding percentages of the 19 month old and the 42 month old samples.

Summary:-

Brain mtDNA isolated from various aged mice was examined by part B of the developed PCR method to confirm the presence of deleted mtDNA molecules. All the products generated by each primer pair combination were synthesized from samples within each age group, indicating that none of the products were age dependent. The only product which displayed both an L strand and a H strand primer shift pattern was the expected 1.6Kb band generated by the primers L567 and H732. When the H primer was changed to H1107, this band showed a corresponding increase in size to 5.4Kb, and shifting the L primer to L480 resulted in the expected 2.5Kb product being amplified. Overall, the smaller amplified DNA products were not age dependent and they did not display a H or an L strand primer shift pattern. This indicates that they do

not represent age related deleted PCR products, but are rather artifacts of the PCR method, such as products resulting from one or both of the primers within each primer pair combination, misannealing to the wildtype DNA template.

4.3. The effect of reducing the PCR extension time on the part A PCR analysis of brain mtDNA, isolated from various aged mice:-

Experimental design:-

Previous studies have shown that a reduction in the extension time increases the amplification of smaller PCR products. Therefore, brain mtDNA from different aged mice (10 weeks old, 19 months old and 42 months old respectively), was re-examined by part A of the developed PCR method using reduced PCR extension times of 40, 20 and 10 seconds respectively. Only the brain mtDNA samples were re-examined by part A using these reduced extension times since the brain is the most likely non-replicative tissue to contain deleted mtDNA molecules. Within any experiment, one brain DNA sample from each age group was analyzed at the same time, and master mixtures of the reaction reagents were prepared where possible. All of the reactions which were to be run with the same extension time were placed in the thermal cycling machine together, and an aliquot of each reaction was subsequently loaded onto a 1% agarose gel.

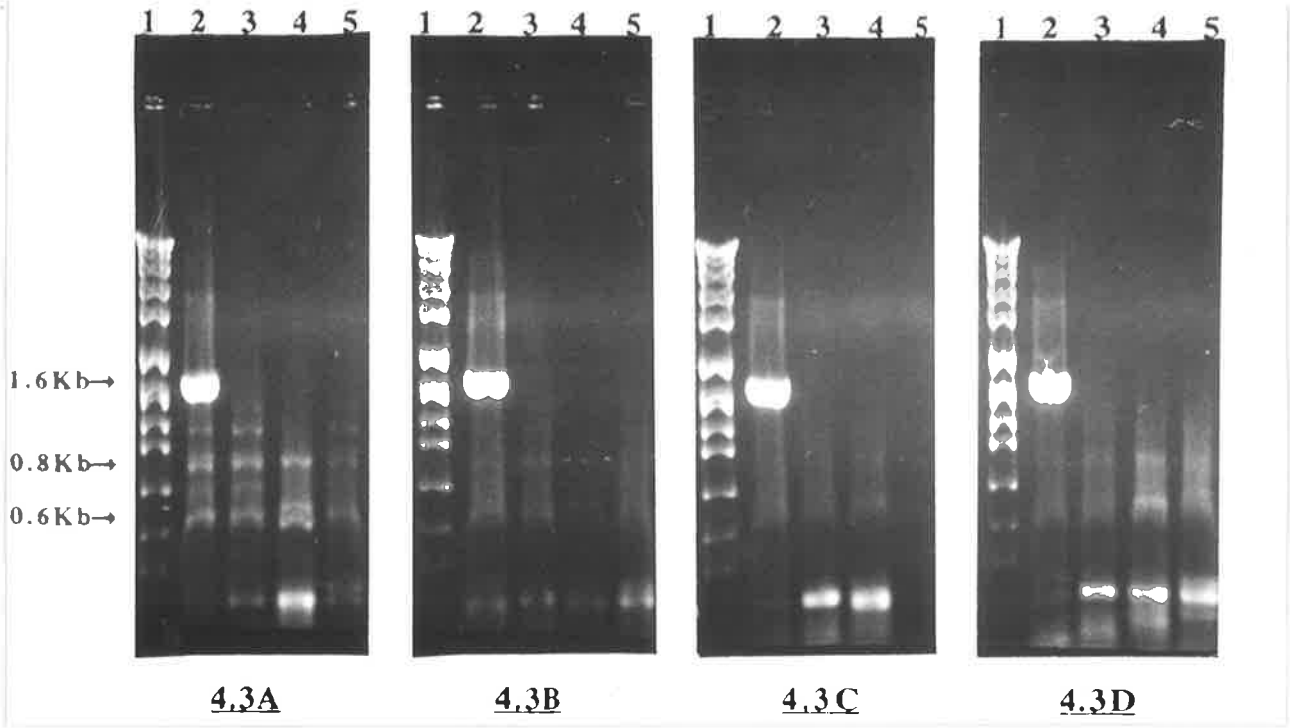
Results:-

The results obtained when a 10 week old brain mtDNA sample was analyzed by part A of the PCR method, with an extension time of 60, 40, 20 and 10 seconds, is

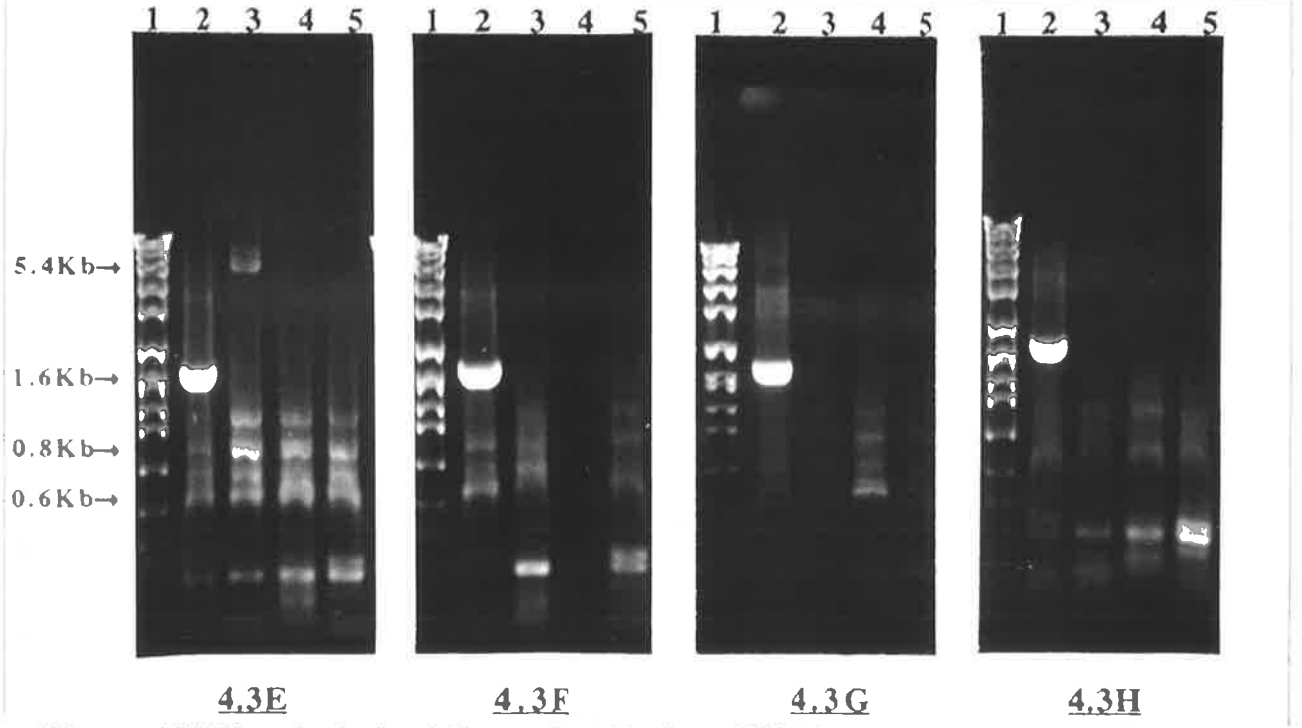
displayed within Figures 4.3A-D respectively. With an extension time of 60 seconds (Figure 4.3A), the same banding pattern which has previously been generated during the part A analysis of 10 week old brain mtDNA was observed. Decreasing the extension time to 40, 20 and subsequently 10 seconds (Figures 4.3B-D), resulted in the products previously generated with an extension time of 60 seconds being no longer detected, except for the expected 1.6Kb PCR product amplified by the primers L567 and H732, which was synthesized regardless of the extension time (lane 2 of each figure), and two products of 0.8Kb and 0.65Kb which were still generated by the primer pair L567-H1295, with an extension time of 10 seconds (Figure 4.3D, lane 4).

Agarose gels showing the results obtained during the PCR analysis of a 19 month old mtDNA sample, are displayed within the Figures 4.3 E-H, which represent different reactions which were performed with extension times of 60, 40, 20 and 10 seconds respectively. Again with an extension time of 60 seconds, the banding pattern observed was typical of that previously generated by the part A PCR analysis of 19 month mtDNA samples (Figure 4.3E). Decreasing the extension time to 40, 20 and eventually 10 seconds (Figures 4.3F-H), resulted in the previously observed bands no longer being detected within the gels, except for the expected L567-H732, 1.6Kb PCR product which was synthesized regardless of the extension time (lane 2 of each figure), and two products of 0.8Kb and 0.65Kb which were still amplified by the primer pair L567-H1295 with extension times of 20 and 10 seconds (Figures 4.3G &H, lane 4).

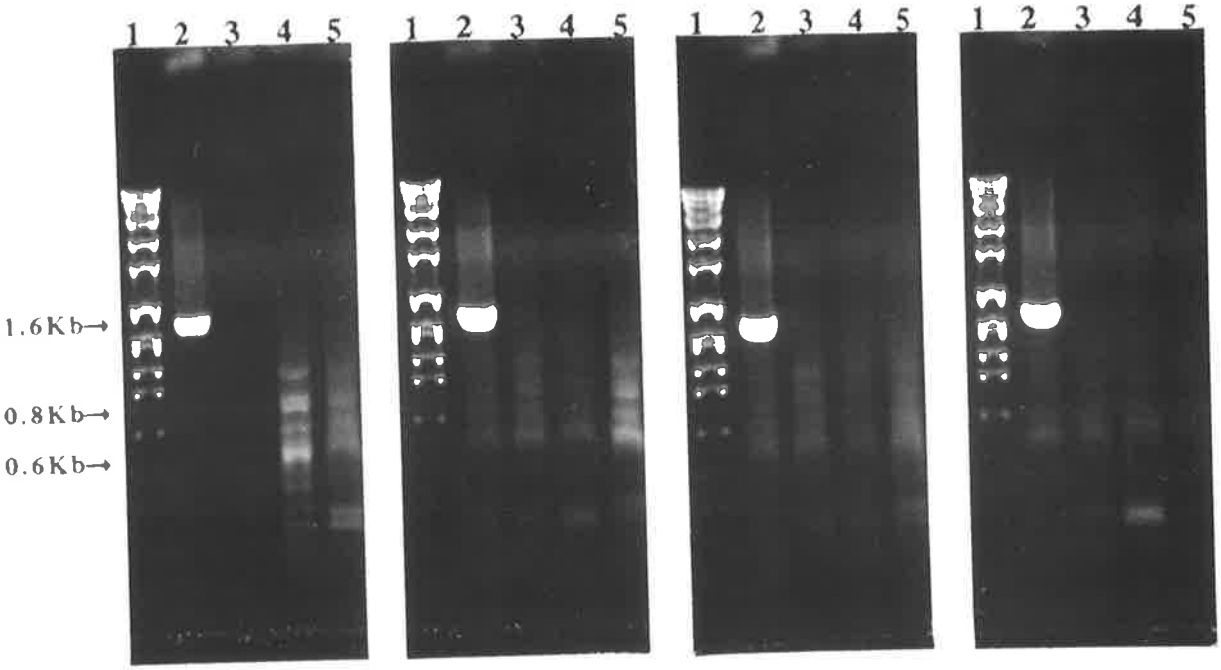
The PCR analysis of a 42 month old mtDNA sample is illustrated within Figures 4.3I-L, which correspond to reactions carried out with extension times of 60, 40, 20 and 10 seconds respectively. The previously synthesized products were again amplified by each primer pair combination with an extension time of 60 seconds (Figure 4.3I). Decreasing the PCR extension time to 40, 20 and subsequently 10 seconds (Figure 4.3J-L), resulted in the bands previously generated by each primer pair no longer being observed within the gel, except for the expected L567-H732, 1.6Kb product which was synthesized within all of the experiments (lane 2 of each figure), and four bands of 1.0Kb, 0.8Kb, 0.7Kb and 0.6Kb which were still amplified by the



Figures 4.3A-D. Analysis of 10 week old brain mtDNA by part A of the developed PCR method using reduced PCR extension times:-
Brain mtDNA isolated from a 10 week old mouse was analyzed by part A of the previously developed PCR method, using different PCR extension times of 60, 40, 20 and 10 seconds (Figures 4.3A, B, C and D respectively). This involved analysis by the following primer pair combinations; L567-H732, L567-H1107, L567-H1295 and L567-H1452 (lanes 2, 3, 4 and 5 respectively within each figure). DNA size markers ranging from 8.5Kb to 0.36Kb were run in lane 1 of each figure.



Figures 4.3E-H. Analysis of 19 month old brain mtDNA by part A of the developed PCR method using reduced PCR extension times:-
Brain mtDNA isolated from a 19 month old mouse was analyzed by part A of the previously developed PCR method, using different PCR extension times of 60, 40, 20 and 10 seconds (Figures 4.3E, F, G and H respectively). This involved analysis by the following primer pair combinations; L567-H732, L567-H1107, L567-H1295 and L567-H1452 (lanes 2, 3, 4 and 5 respectively within each figure). DNA size markers ranging from 8.5Kb to 0.36Kb were run in lane of each figure.



Figures 4.3I-L. Analysis of 42 month old brain mtDNA by part A of the developed PCR method using reduced PCR extension times:-
Brain mtDNA isolated from a 42 month old mouse was analyzed by part A of the previously developed PCR method, using different PCR extension times of 60, 40, 20 and 10 seconds (Figures 4.3I, J, K and L respectively). This involved analysis by the following primer pair combinations; L567-H732, L567-H1107, L567-H1295 and L567-H1452 (lanes 2, 3, 4 and 5 respectively within each figure). DNA size markers ranging from 8.5Kb to 0.36Kb were run in lane 1 of each figure.

primer pair L567-H1452 with an extension time of 40 seconds (Figure 4.3J, lane 5).

Summary:-

Reducing the PCR extension time from 60 seconds to 40, 20 and subsequently 10 seconds, did not enhance the production of any new DNA fragments which might represent age dependent, deleted PCR products. Infact, shortening the time required for the extension of a PCR product, resulted in the majority of the previously synthesized bands no longer being generated. The expected L567-H732, PCR product of 1.6Kb was amplified within all the experiments, regardless of the PCR extension time, which indicates that an extension time of 10 seconds is sufficient time to amplify a 1.6Kb product. Overall, no primer shift patterns were observed within each age group, and no age specific products were detected.

4.4. The effect of increasing the number of PCR cycles on the part A PCR analysis of brain mtDNA isolated from various aged mice:

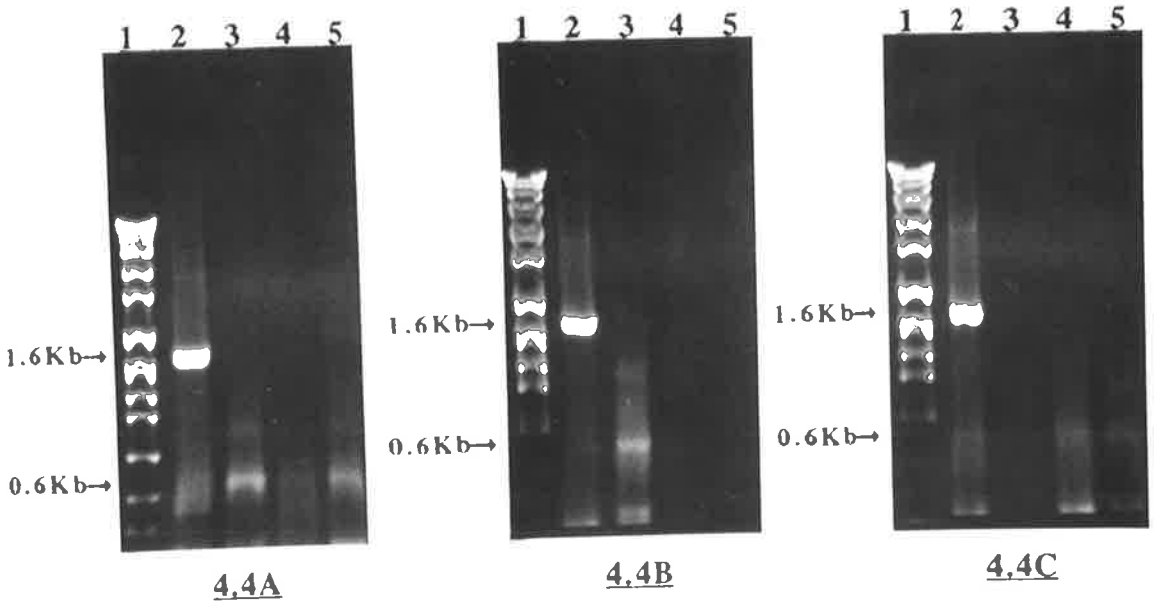
Experimental design:-

One feature of the PCR technique is that the DNA template can be amplified to a greater extent by increasing the number of PCR cycles, provided that the reaction reagents are continuously replenished. Therefore, since the number of deleted mitochondrial genomes may be too low to be successfully amplified after 40 cycles, brain mtDNA from different aged mice (10 weeks old, 19 months old and 42 months

old respectively), was re-examined by part A of the developed PCR method for a total of 80 PCR cycles. Only the brain mtDNA samples were analyzed since the brain is the most likely non-replicative tissue to contain deleted mtDNA molecules. Within each experiment, at least one brain mtDNA sample from each age group was analyzed at the same time. Master mixtures of the reaction reagents were prepared where possible. The normal PCR reactions of 40 cycles was initially performed for each of the four primer pairs of part A of the developed PCR method. An aliquot from each tube was subsequently removed and diluted 1 in 10 000, to be used as the DNA template for a second 40 cycle PCR reaction. The second PCR reactions were prepared using identical reaction reagents as the first reactions, except the original DNA template was omitted, and was replaced with the respective diluted aliquot from each of the initial PCR reactions. These second PCR reactions were performed for 40 cycles, resulting in an overall 80 cycle PCR amplification of each mtDNA sample by each of the primer pair combinations.

Results:-

The results obtained from the 80 cycle PCR analysis of various aged (10 weeks old, 19 months old and 42 months old) brain mtDNA samples can be seen within Figures 4.4A-C respectively. The expected PCR product of 1.6Kb was amplified by the primer pair L567-H732 from samples within each age group (lane 2 of each figure). The primer pair L567-H1107, resulted in the synthesis of a band of approximately 0.6Kb being observed following the analysis of the 10 week old and the 19 month old mtDNA samples (Figures 4.4A & B, lane 3), whilst no products were synthesized from the 42 month old sample by this primer pair (Figure 4.4C, lane 3). PCR analysis with the primers L567 and H1295 did not generate any products from any of the samples (lane 4 of each figure), however, shifting the H primer to H1452 (lane 5 of each figure), resulted in a band of approximately 0.6Kb being observed following the



Figures 4.4A-C. Part A PCR analysis of various aged brain mtDNA for a total of 80 PCR cycles:-
Brain mtDNA from a 10 week old, a 19 month old and a 42 month old mouse were analyzed by part A of the developed PCR method, for a total of 80 PCR thermal cycles (Figures 4.4A, B & C respectively). This involved the analysis of each sample by the following primer pair combinations; L567-H732, L567-H1107, L567-H1295 and L567-H1452 (lanes 2, 3, 4 and 5 respectively in each figure). DNA size markers ranging from 8.5Kb to 0.36Kb were run in lane 1 of each figure.

analysis of the 10 week old sample (Figure 4.4A).

Summary:-

Increasing the number of PCR cycles to 80 cycles, did not enhance the production of any smaller, deleted PCR products by the part A PCR analysis of brain mtDNA. Infact, nearly all the bands previously observed after 40 cycles were no longer present within the gels. Only a 0.6Kb product was amplified from the 10 week old and the 19 month old samples by the primers L567 and H1107, while this same size band was also observed following the analysis of the 10 week old sample by the primer pair combination L567-H1452. Therefore, overall, no age dependent bands were observed, showing a primer shift pattern.

4.5. Conclusions:-

Analysis of brain, heart, skeletal muscle and liver mtDNA from various aged mice, by part A of the developed PCR method did not generate any age dependent, or tissue specific deleted PCR products which displayed a H strand primer shift pattern. This implies that the bands observed within each gel were most likely artifacts of the PCR technique, which may represent non-specific PCR products resulting from the primers misannealing to the wildtype DNA template. The only product which displayed this H strand primer shift pattern was the expected PCR product synthesized by the primers L567 and H732.

Further analysis of the different aged brain samples by part B of the developed PCR method also resulted in no age dependent deleted PCR products being amplified, nor did any of the bands display an L or a H strand primer shift pattern. This confirms

that the bands generated by each primer pair were not genuine deleted PCR products. Again the only product which displayed both primer shift patterns was the expected 1.6Kb product generated by the primers L567-H732 from the wildtype mtDNA template.

To enhance the synthesis of the smaller deleted PCR products, the PCR extension time was reduced and to increase the amplification of these rarer deleted mtDNA genomes, the number of PCR amplification cycles was doubled. However, both of these procedures failed to generate any deleted PCR products.

Chapter 5:

Direct repeat sequences within the mouse mitochondrial genome

5.0. Introduction:-

The majority of the DNA deletions discovered within the human mitochondrial genome have been found to lie within the major region between the two origins of replication, and in between two tandem direct repeat sequences. The sequence of the most common human deletion of 4.977Kb, has revealed that this deletion is flanked by two 13bp direct repeat sequences which are located within the ATPase 8 gene and the ND5 gene. Therefore, in an effort to predict the location of deletions within the ageing mouse, tandem direct repeat sequences within the mouse mitochondrial genome were analyzed. This search was limited to those repeats which were 10bp or greater since larger direct repeats of 13bp and 12bp have been associated with the more common deletions of 4.977Kb and 7.436Kb respectively within the human genome (Cortopassi & Arnheim 1990, Hattori et al 1991b). The gene position of each of these 10bp repeats was compared to the position of the 13bp repeat which surrounds the most common human deletion to determine if this repeat is conserved between species, which would indicate whether this same deletion may also exist within the ageing mouse genome. Those repeat sequences of the mouse which shared the same gene locations as the human 13bp repeat were then analyzed and the % homology which they shared with the human repeat was calculated.

The position of all the direct repeats of 10bp or greater, which were confined to the major region between the two origins of replication, were also compared to the positions of the primers used in the previously developed two part PCR method, for detecting mouse mtDNA deletions. As a result, the different aged brain mtDNA

samples were re-analyzed by a modification of the original PCR method, to enhance the amplification of segments of the mitochondrial genome, within the major region between the replication origins, which contained direct repeat sequences which would not have been amplified by any of the primer pair combinations previously used.

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

Experimental design:-

All the direct repeat sequences of 10 basepairs or greater within the mouse mtDNA genome were identified and examined. Following the analysis of all the repeats which were confined to the major region between the two origins of replication, a list of those direct repeats which had their repeat sequences positioned at least 3.5Kb apart was constructed, to subsequently determine those direct repeats which were located within both the ATPase 8 and the ND5 genes (which are 3.7Kb apart), which is where the 13bp direct repeat which surrounds the most common human deletion is positioned. The sequences of these mouse repeats were then compared with the sequence of the human 13bp repeat and the percent homology was calculated.

Results:-

A complete list of all the direct repeats within the mouse mitochondrial genome, which are 10bp or greater, is shown in Table 5.1.1. In total, 480 direct repeats were discovered dispersed throughout the entire genome, ranging from 10bp to 16bp in size. A summary of the size and position of each of these relative to the two origins of

Table 5.1.1. Direct repeat sequences within the mouse mitochondrial genome:
This table lists all the direct repeat sequences within the mouse mitochondrial genome which are 10bp or greater. The nucleotide positions, size (bp) and sequence (L strand) of each of these repeats is displayed in accordance with the sequence of the mouse mitochondrial genome derived by Bibb et al (1981).

Nucleotide position:		Size/Sequence: (bp)	
8668	10486	10	CCTTTTCAGC
10358	12551	10	TATTTTAACA
599	13494	11	TACTTTATATC
2958	9991	11	TTATTTATTAT
6358	11313	10	TTATTTTCTT
6366	12591	10	TTATTTACAG
10357	12550	11	TTATTTTAACA
4166	12144	10	ATATTTCAAC
9487	10516	10	ATATTTTATT
14694	15174	12	CCATTTATTATC
6599	10173	12	ATTCTTCCCTCA
10860	11493	10	ATTCTTCTAA
13520	15221	10	CATCTTAATT
2954	9987	15	ATCCTTATTTATTAT
8666	14321	10	AGCCTTTTCA
598	13493	12	GTACTTTATATC
11217	12369	10	CCACTTATAG
3240	8427	10	ACACTTATTA
3240	10545	10	ACACTTATTA
8427	10545	10	ACACTTATTA
11958	14036	10	GAACTTAAAA
8	1032	10	TAGCTTAATA
6176	6353	10	AGGCTTTATT
2957	9990	12	CTTATTTATTAT
4295	11490	11	CTTATTCTTCT
8347	10548	12	CTTATTATTATT
4538	11337	10	ATTATTCTTA
10551	11358	11	ATTATTATTAC
11294	14056	11	ATTATTCATTA
3134	5387	12	ACTATTCGGAGC
10322	12338	10	TATATTCTCC
10508	10515	10	TATATTTTAT
7947	14186	10	CTCATTCATT
6518	14162	10	CCCATTATTT
11811	15398	10	CTAATTAAAC
90	943	10	ATAATTAATT
7097	8137	10	ATAATTGTTT
1471	4331	10	TCAATTTTAA
6957	9033	10	TCAATTACAT
3742	6981	11	TAAATTATAGA
10467	11855	11	CAAATTCTCCT
385	7918	11	AGAATTAAAAT
4252	12108	10	CTGATTACCA
3302	7789	10	ATGATTTATC
15332	15637	10	TCTTCTCTTC
4606	10280	10	ACTTCTATGA
10859	11492	11	TATTCTTCTAA
3536	4768	10	AGCTCTACTA
10651	13519	10	TCATCTTAAT
10651	15220	10	TCATCTTAAT
13519	15220	11	TCATCTTAATT
4530	6996	10	TAATCTATAT
10604	14957	10	TTTCCTATTT
2953	9986	16	TATCCTTATTTATTAT
10943	13294	10	CATCCTTCTC
10014	12302	10	TTCCCTAAAC
699	13928	10	AACCCTAAAA
9089	10095	10	AGCCCTACTA
9089	12956	15	AGCCCTACTAATTAC
10095	12956	10	AGCCCTACTA
3985	4879	10	CAACCTAATA
4522	13680	10	CAACCTCATA
120	13976	11	AAACCTCCATA
14470	15316	10	AAACCTGAAA
808	5943	10	ATTACTATAC
3511	4516	10	TCTACTCAAC
9092	12191	10	CCTACTAATT
9092	12959	12	CCTACTAATTAC
12191	12959	10	CCTACTAATT
10190	13601	10	GCTACTACCA
11374	13537	10	TATACTCAAT
3991	11099	10	AATACTAATA
13784	15263	10	AATACTAAAA
9011	14486	10	AGTACTTCTA
4813	6707	10	TTCACTAACA
10913	13957	11	CCCACTAACAA
3239	10544	11	AACACTTATTA
1711	12544	10	ATAACTTTAT
365	4140	11	TCAAGTATAAA
3118	11149	10	CAAACTCCAA
10802	12428	10	ATGACTACCA
10637	11623	10	ACTGCTAATT
7	1031	11	GTAGCTTAATA
8452	15360	10	GGAGCTACTC
6175	6352	11	TAGGCTTTATT
2960	8502	10	ATTTATTATT
14696	15176	10	ATTTATTATC
2956	9989	13	CCTTATTTATTAT
3242	5891	10	ACTTATTACA
4294	11489	12	ACTTATTCTTCT
8346	10547	13	ACTTATTATTATT
9930	16203	10	ACTTATATTT
3191	9983	10	TATTATCCTT
8349	10550	10	TATTATTATT
13529	15561	10	TATTATCTTA
3440	10901	10	CATTATTCTA
6520	11311	10	CATTATTTTC
4444	9719	11	AATTATAGCCT
6083	13208	10	AATTATTTCA
11293	14055	12	AATTATTCATTA
11293	16030	10	AATTATTCAT
14055	16030	10	AATTATTCAT
3613	4608	10	TTCTATGAAA
4582	12252	10	CTCTATAACC
6251	8712	10	CACTATAATT
603	15273	10	TTATATCCAT
10507	10514	11	TTATATTTTAT
10507	16205	10	TTATATTTTA
10514	16205	10	TTATATTTTA
12053	14378	10	ATATATACAC
1037	6317	10	TAATATTAAA
3298	8701	11	TAATATGATTT
7143	16147	10	TAATATTAAC
1119	14049	12	AAATATAATTAT
6810	9197	10	CTTCATGGCT
7950	11297	10	ATTCATTACC
11630	16034	10	ATTCATGCTT
9101	10780	10	TACCATTATA
3182	7619	10	AACCATAGCT
7894	13256	10	AACCATAAAA
7779	13687	10	ATACATCAAC
15460	15495	10	GTACATTAAA
11122	12838	11	TCACATCATCA
3437	11354	10	TAACATTATT
3553	9241	10	CAACATTCCT
13660	15881	10	CAACATAACT
3212	8466	10	ATTAATAAAT
9483	11888	11	ATTAATATTTT
8148	10475	10	CCTAATCATA
8148	14834	12	CCTAATCATATT
10475	14834	10	CCTAATCATA
12914	13205	10	CCTAATTATT
4939	9095	10	ACTAATTACC
5579	11102	11	ACTAATAATCG
11012	12971	10	ACTAATCGCC
11516	12962	10	ACTAATTACA
8100	14913	10	GCTAATCCAC
89	9401	10	TATAATTAAT
4849	12540	10	AATAATAACT
7096	8136	11	AATAATTGTTT
9716	10964	10	AATAATTATA
8720	9032	10	TTCAATTACA
8523	9740	10	CACAATTCTA
9695	13963	10	AACAATTAAA
2174	6980	10	TTAAATTATA
244	8470	10	ATAAATATTA
371	375	11	ATAAATAAATA
9555	13281	12	GCAAATCCATAT
1650	9274	10	TAAAATTTCA
2908	14171	10	TAAAATTATT
4477	15556	10	ATGAATATTA
6629	12878	10	AGGAATACCA
8162	9235	11	TTGGATCAACA
4686	8890	10	CAGGATTCTT
6499	13126	10	CAGGATTTGT
9810	10260	10	ATTAGTTTAA
6860	7900	10	AAAAGTAAAA
2726	3750	10	AGAGGTTCAA
790	6360	10	ATTTTCTTAT
7833	12145	10	TATTTCAACT
10602	14955	12	TATTTCCTATTT
7695	8328	10	AATTTCACTA
6600	10174	11	TTCTTCCCTCA
8513	14488	10	TACTTCTACT
4605	10279	11	CACTTCTATGA
4296	11491	10	TTATTCTTCT
11295	14057	10	TTATTCATTA
3135	5388	11	CTATTCGGAGC
4241	8240	10	CCATTCCACT
447	9508	10	TAATTCTAGT
11482	12364	13	TAATTCCACTTAT
10468	11856	10	AAATTCTCCT
8047	9365	10	CGTCTCCATT
1948	5716	10	CACCTCTAGC
121	13977	10	AACCTCCATA
2945	13765	10	AACCTCTATA
4523	10683	11	AACCTCATAAT
4108	14396	10	AGCCTCAATA
3512	4355	10	CTACTCAACT
10020	11150	11	AAACTCCAACT
10941	13292	12	TTCATCCTTCTC
3548	15528	11	ATCATCAACAT
13518	15219	12	ATCATCTTAATT
9126	15685	10	ACCATCCTCC
11123	12839	10	CACATCATCA
2790	12972	10	CTAATCGCCA
4043	8035	11	CTAATCAACAA
8149	14835	11	CTAATCATATT
4529	6995	11	ATAATCTATAT
7548	9082	10	TAAATCAAGC

Nucleotide position:		Size/Sequence: (bp)	
2955	9988	14	TCCTTATTTATTAT
1690	11959	10	AACTTAAAAT
2707	7518	10	GACTTAAAAC
5818	11392	10	TTATTACCAC
8348	10549	11	TTATTATTATT
10552	11359	10	TTATTATTAC
3030	10350	10	CCATTAATTA
10900	11335	10	CCATTATTCT
806	9958	10	ACATTACTAT
15462	15574	11	ACATTAAACTA
529	13483	10	TAATTAAATT
944	3034	10	TAATTAATTT
3857	5069	11	TAATTAAGCTA
4740	9718	10	TAATTATAGC
8264	11518	11	TAATTACAGGC
9808	10258	12	TAATTAGTTTAA
14054	16029	11	TAATTATTCAT
3743	6982	10	AAATTATAGA
13755	13890	13	AAATTAAAAAAAC
386	7919	10	GAATTAAAAT
1301	11496	10	CTTCTAACTA
3612	4607	11	CTTCTATGAAA
449	9732	10	ATTCTAGTCA
2947	7126	10	CCTCTATATC
3509	4514	12	ACTCTACTCAAC
2189	2542	10	TATCTATTTA
7106	13506	10	TTCCTAATTA
11790	15030	10	ATCCTAATTT
9090	12957	14	GCCCTACTAATTAC
3543	4365	10	CTACTATCAT
9093	12960	11	CTACTAATTAC
4040	10452	10	ATACTAATCA
4814	7886	10	TCACTAACAA
10914	13958	10	CCACTAACAA
2988	3639	12	ACACTAGCATTA
366	4141	10	CAACTATAAA
2844	11156	10	CAACTACGAA
337	11514	10	AAACTAATTA
4445	9720	10	ATTATAGCCT
368	1829	11	ACTATAAATAA
13141	16143	11	CATATAATATT
1120	14050	11	AATATAATTAT
12052	14377	11	GATATATACAC
4199	6591	11	AACATAACATT
9484	11889	10	TTAATATTTT
5580	11103	10	CTAATAATCG
7094	8134	13	CTAATAATTGTTT
7142	10383	10	CTAATATTAA
2143	13565	10	ATAATAATAA
9714	13738	10	ATAATAATTA
4112	7811	10	TCAATAATTA
11737	14400	10	TCAATATTTT
372	376	10	TAAATAAATA
2639	10922	10	AAAATATATA
7321	9323	10	ATGATACTGA
2310	6861	10	AAAGTAAAAT
6705	8329	10	ATTTCACTAA
7696	8708	10	ATTTCACTAT
6756	11971	10	GCTTCAAAAC
669	4014	11	AATTCAGCCTA
6934	9606	10	TTCTCAATAA
8758	13171	11	TCCTCACAATA
651	9893	10	ACCTCACCAT
4524	10684	10	ACCTCATAAT
3513	4137	10	TACTCAACTA
3549	15529	10	TCATCAACAT
3549	15932	10	TCATCAACAT
6724	14220	10	TCATCATGAT
15529	15932	11	TCATCAACATA
4044	8036	10	TAATCAACAA
8150	14836	10	TAATCATATT
7883	11007	10	AAATCACTAA
4243	4806	10	ATTCCACTTC
11484	12366	11	ATTCCACTTAT
4218.	8219	10	TATCCATAAA
1059	6045	10	CACCCAGAAG
13089	16011	10	AACCCAATCA
9100	11332	10	TTACCATTAT
1595	1790	10	TAACCATTGT
3181	7618	11	TAACCATAGCT
4830	13946	10	CAACCAACAA
13465	14018	14	CAACCAACCAAAAA
13469	14022	10	CAACCAAAAA
13750	15098	10	CACACAAATT
538	1579	10	TTAACAAAAC
1644	15532	10	TCAACATAAA
3552	9240	11	TCAACATTCCT
4833	7960	10	CCAACAATAA
13659	15880	11	CCAACATAACT
1941	9293	10	AAAACATCAC
8886	13122	11	TTTGCAGGATT
4025	4382	10	CTAGCAATTA
4100	7553	10	CAAGCAACAG
2938	13459	11	CTTTAACAACC
3040	5273	10	ATTTAAACCT
14845	15223	10	TCTTAATTCT
158	1691	10	ACTTAAAATT
2202	13903	12	TATTAAACCTAA
3211	8465	11	TATTAATAAAT
15463	15575	10	CATTAAACTA
3858	5070	10	AATTAAGCTA
5102	13966	11	AATTAAACCTA
13756	13891	12	AATTAAAAAAAC
10474	14833	11	TCCTAATCATA
1349	13909	10	ACCTAAAAAC
3824	5968	10	ACCTAAACAC
9094	12961	10	TACTAATTAC
4815	13959	10	CACTAACAAT
5578	7092	10	CACTAATAAT
11011	12970	11	CACTAATCGCC
338	10501	10	AACTAATTAT
4938	11515	10	AACTAATTAC
665	11625	10	TGCTAATTCA
474	5075	10	AGCTAAGACC
796	2917	12	TTATAAAAGAAC
369	1830	10	CTATAAATAA
1121	14051	10	ATATAATTAT
13142	16144	10	ATATAATATT
4221	13258	11	CCATAAAACTA
4200	6592	10	ACATAACATT
9079	15535	11	ACATAAATCAA
7095	8135	12	TAATAATTGTTT
10146	13358	10	TCTCAACCTA
15530	15933	10	CATCAACATA
7279	8037	11	AATCAACAACC
1450	16115	10	ACCCAAAAAA
13466	14019	13	AACCAACCAAAAA
3247	4498	10	TTACAACCCA
8522	9739	11	TCACAATTCTA
3460	12556	10	TAACAACTAT
4383	9628	10	TAGCAATTAC
9553	13279	14	AAGCAAATCCATAT
2203	5103	10	ATTAAACCTA
2203	13904	11	ATTAAACCTAA
2203	13967	11	ATTAAACCTAA
5103	13904	10	ATTAAACCTA
5103	13967	10	ATTAAACCTA
13757	13892	11	ATTAAAAAAAC
13904	13967	12	ATTAAACCTAAA
1350	13370	10	CCTAAAAACA
10218	10409	10	CCTAAAAAAA
10679	13973	10	CCTAAACCTC
797	2918	11	TATAAAAGAAC
4222	13259	10	CATAAAACTA
9080	15536	10	CATAAATCAA
374	13271	10	AATAAATAAA
2312	6950	11	AGTAAAATCAA
1143	9290	10	ATCAAAACAT
540	7153	10	AACAAAACTA
9554	13280	13	AGCAAATCCATAT
13758	13893	10	TTAAAAAAAC
1486	10219	10	CTAAAAAAAC
798	2919	10	ATAAAAGAAC
7898	11727	10	ATAAAAGTAA
2313	6951	10	GTAAAATCAA
1497	16232	10	ACAAAATCAA
4082	15259	10	ACAAAATACT
8831	9776	10	ACAAAAAGGA
10220	13759	11	TAAAAAAACCT
4845	14026	10	CAAAAATAAT
1489	4726	10	AAAAAACAAC
4054	13790	10	AAAAAACCCA
5172	5173	10	AAAAAAAAAA
10221	13760	10	AAAAAAACCT
4161	15555	10	CATGAATATT
1311	3722	10	AAAGAATTAC
6628	12877	11	CAGGAATACCA
4160	5236	10	ACATGAATAT
4436	14148	10	ATACGAAAAA
7447	11954	10	AATAGAACTT
2336	5594	10	CCCAGATATA
5363	14816	10	CAAAGATATC
1310	3721	11	AAAAGAATTAC
8161	9234	12	ATTGGATCAACA
6498	13125	11	GCAGGATTTGT
8884	13120	13	TCTTTGCAGGATT
8159	9232	14	TTATTGGATCAACA
5564	11977	10	AAACTGACTT
9562	9763	10	CATATGAATG
3299	8702	10	AATATGATTT
1281	14031	10	ATAATGAACT
1456	4699	10	AAAATGAATT
3136	5389	10	TATTCGGAGC
5899	11176	10	CAGCCGTACT
9809	10259	11	AATTAGTTTAA
4023	4380	12	TACTAGCAATTA
2989	3640	11	CACTAGCATTA
4227	12661	10	AACTAGGCCT
2797	9899	11	CCATAGCCTTC
4209	11467	10	TAATAGCCCT
7446	11953	11	CAATAGAACTT
670	4015	10	ATTCAGCCTA
1060	9444	10	ACCCAGAAGA
8887	13123	10	TTGCAGGATT
9085	10211	11	ATCAAGCCCTA
1309	3720	12	TAAAAGAATTAC
6859	7899	11	TAAAAGTAAAA
6556	10810	10	CAAAAGCCCA
1894	10414	10	AAAAAGATAA
8160	9233	13	TATTGGATCAACA

Nucleotide position:		Size/Sequence: (bp)	
9556	13282	11	CAAATCCATAT
7882	11006	11	AAAATCACTAA
3595	4063	10	ACGATCAACT
8163	9236	10	TGGATCAACA
2089	6681	10	ACTGTCTCTT
10603	14956	11	ATTTCCTATTT
6601	10175	10	TCTTCCCTCA
11483	12365	12	AATTCCACTTAT
10896	13056	10	ATCTCCATTA
8819	11131	10	CACTCCTATT
10021	11151	10	AACTCCAACT
4217	8218	11	CTATCCATAAA
10942	13293	11	TCATCCTTCTC
7377	10537	11	TAATCCCAACA
2385	8009	10	CAATCCTATT
9557	13283	10	AAATCCATAT
7378	10538	10	AATCCCAACA
913	8567	10	TCACCCTCCT
12524	16092	10	CCACCCCCTC
3026	14159	10	ACACCCATTA
698	13927	11	AAACCCTAAAA
13088	16010	11	AAACCCAATCA
13793	16113	10	AAACCCAAAA
479	12065	10	AGACCCAAAC
12431	13604	10	ACTACCATCA
3025	14158	11	CACACCCATTA
4521	13679	11	TCAACCTCATA
5352	13692	10	TCAACCAATC
3984	4878	11	CCAACCTAATA
13468	14021	11	CCAACCAAAAA
13464	14017	15	ACAACCAACCAAAAA
13868	13945	10	ACAACCAACA
519	13906	10	TAAACCTAAA
519	13969	10	TAAACCTAAA
13906	13969	10	TAAACCTAAA
697	13926	12	CAAACCCTAAAA
9702	13763	10	AAAACCTCTA
1107	10093	10	CTAGCCCTAC
5898	11173	11	ACAGCCGTACT
8740	15921	10	TTGGCCTACT
807	5942	11	CATTACTATAC
8265	11519	10	AATTACAGGC
8723	12965	10	AATTACACTA
3510	4515	11	CTCTACTCAAC
9091	12958	13	CCCTACTAATTAC
9581	12238	10	CCCTACAAGC
10450	11098	10	CAATACTAAT
8759	13172	10	CCTCACAATA
4807	14679	10	TTCCACTTCA
6456	8243	10	TTCCACTATG
11485	12367	10	TTCCACTTAT
10912	13956	12	ACCCACTAACAA
8725	12097	10	TTACACTATT
8425	9926	10	GGACACTTAT
4909	7171	10	AAGCACAATA
2939	13460	10	TTTAACAACC
937	4196	10	CTTAACATAA
3551	9239	12	ATCAACATTCCT
5832	7783	10	ATCAACATGA
7280	8038	10	ATCAACAACC
13467	14020	12	ACCAACCAAAAA
1493	7150	10	AACAACAAAA
13463	13944	10	AACAACCAAC
2204	13905	10	TTAAACCTAA
2204	13968	10	TTAAACCTAA
11815	15568	10	TTAAACACAT
13905	13968	11	TTAAACCTAAA
15294	16282	10	ATAAACATTA
3117	5864	10	TCAAACTCCA
4426	13862	10	CCAAACACAA
1090	10498	10	CTGAACTAAT
1763	14877	10	CCAGACATAC
8781	14349	10	CGAGACGTAA
8885	13121	12	CTTTGCAGGATT
511	14288	10	CTTAGCCATA
1100	1106	10	CCTAGCCCTA
12704	13103	10	CCTAGCATTC
2990	3641	10	ACTAGCATTA
4024	4381	11	ACTAGCAATTA
2798	9900	10	CATAGCCTTC
4561	11039	10	CATAGCACTT
6	1030	12	TGTAGCTTAATA
9086	10212	10	TCAAGCCCTA
8451	15359	11	AGGAGCTACTC
4334	5430	10	ATTTTAATTC
600	13495	10	ACTTTATATC
2959	9992	10	TATTTATTAT
8501	14695	10	CATTTATTAT
8501	15175	10	CATTTATTAT
14695	15175	11	CATTTATTATC

replication can be seen in Table 5.1.2. Only one 16bp direct repeat was detected which had one repeat sequence located within the major region between the two origins of replication and the other sequence positioned within the minor region. A total of three 15bp direct repeats were discovered, where two of these had both of their repeat sequences confined to the major region, while the third had one repeat sequence within each region.

Five 14bp direct repeats were found, of which four had both of their repeat sequences localized to the major region, and the fifth had one repeat sequence located within each region. In total, ten 13bp direct repeats were discovered where 9 were confined to the major region, and the tenth had one repeat sequence located within each region. Of the 33 12bp repeat sequences, 19 were confined to the major region, while 6 were confined to the minor region, and the remaining 8 had one of their repeat sequences located within each area. A total of 98 11bp direct repeat sequences were identified, with 53 being located entirely within the major region, 13 were confined to the minor region, and 32 had one repeat sequence positioned within each area. Of the 330 10bp direct repeat sequences, 158 had both of their repeat sequences confined to the major area, while 36 were confined to the minor region, and 136 of these repeats had one repeat sequence located within each area.

Overall, in total there were 245 direct repeats confined to the major area between the two origins of replication, 55 repeat sequences positioned within the minor region, and a total of 180 direct repeats which had one repeat sequence located within each region. In comparison to the number of direct repeats found within the human mtDNA genome, there were more repeats of each repeat size found within the mouse mtDNA, with a total of 480 being identified, whilst only 332 direct repeats ranging from 10-15bp were detected within the human genome by Schon et al (1989).

Since the ATPase 8 and the ND5 genes are positioned 3.7Kb apart, and these genes have been found to house the 13bp direct repeat which surrounds the most common deletion within the human genome, a list was constructed from the 245 repeats

Table 5.1.2. Direct repeat sequences of 10bp or greater within the mouse mtDNA genome:

This table summarizes all the direct repeats within the mouse mitochondrial genome which are 10bp or greater. The size of the repeat and the relative position of each compared to the two origins of replication is listed. Major = The number of different direct repeats where both of the repeat sequences are located within the major region between the two origins of replication, Minor = The number of direct repeats where both of the repeat sequences are located within the minor region between the two origins of replication, Major/Minor = The number of different direct repeats where one repeat sequence is located within the major region, and the other is positioned within the minor region between the two origins of replication. For comparison, the number of different repeats of each size is also listed for the human mtDNA genome which were discovered by Schon et al (1989).

Repeat size:	Position			Total	Total
	Major:	Minor:	Major/Minor:	Mouse:	Human:
16bp	-	-	1	1	-
15bp	2	-	1	3	1
14bp	4	-	1	5	-
13bp	9	-	1	10	4
12bp	19	6	8	33	16
11bp	53	13	32	98	58
10bp	158	36	136	330	253
Total:	245	55	180	480	332

which are confined to the major region, of those repeats which had their repeat sequences positioned at least 3.5Kb apart (Table 5.1.3). This table lists a total of 85 different repeats which could theoretically generate deletions ranging from 3.539Kb to 9.453Kb. For example, one of the 10bp direct repeats which is positioned at 6358 and 11313 would theoretically generate a deletion of 4.955Kb which is similar in size to the most common 4.977Kb deletion discovered within the human genome.

From this list, only 2 of these direct repeats were found to display the same gene locations as the 13bp direct repeat which surrounds the most common human mtDNA deletion. The sequences and gene positions of each of these direct repeats are shown in Table 5.1.4. These two different direct repeats both contain 10 nucleotide bases, where one of their repeat sequences is located within the ATPase 8 gene at the nucleotide position 7833 and 7894 respectively, while their other sequence is positioned within the ND5 gene at the nucleotide position 12145 and 13256 respectively. Thus, if either of these direct repeats represent a deletion 'hot spot' within the mouse mitochondrial genome, they would have the potential to generate deletions of 4.312Kb and 5.362Kb respectively. The greatest percentage homology each of these two 10bp sequences share with any 10 consecutive bases of the human 13bp direct repeat was calculated to be 50% and 40% respectively.

The position of the 245 direct repeats which are confined to the major region between the two origins of replication were also examined for those repeats which had one repeat sequence located within the ATPase 6 gene and the other in the ND5 gene, since a deletion bounded by two 16bp direct repeats located within these two genes has previously been discovered within the ageing rat. However, this comparison was negative: it was not possible to find two mouse direct repeats mapping in these same two gene locations.

Finally, following the analysis of the 85 direct repeats which are located within the major region between the two origins of replication and have repeat sequences which are at least 3.5Kb apart, it was found that the majority of these were positioned within the confines of the primers utilized within the two part PCR method previously

Table 5.1.3. Mouse mtDNA direct repeats:
This table summarises all the direct repeats within the mouse mtDNA genome of 10bp or greater, which are located entirely within the major region between the two origins of replication, and have repeat sequences which are at least 3.5Kb apart. This table also lists the theoretical deletion size (Kb) which would be generated from each of these repeats.

Position:	deletion (Kb):
15bp	
9089 - 12956	3.867
14bp	
9090 - 12957	3.867
9553 - 13279	3.726
13bp	
9091 - 12958	3.867
9554 - 13280	3.726
8884 - 13120	4.236
12bp	
9092 - 12953	3.726
8148 - 14834	6.691
9555 - 13281	3.726
10602 - 14955	4.353
8885 - 13121	4.236
6599 - 10173	3.574
11bp	
5579 - 11102	5.523
6600 - 10174	3.574
8149 - 14835	6.691
9556 - 13282	3.726
10603 - 14956	4.353
5898 - 13173	7.275
8451 - 15359	6.908
9093 - 12960	3.867
8758 - 13171	4.413
8886 - 13122	4.236
10474 - 14833	4.359
9079 - 15535	6.456
10220 - 13759	3.539
6628 - 12877	6.249
6498 - 13125	6.627
7446 - 11953	4.507
10bp	
6358 - 11313	4.955
6366 - 12591	6.225
8666 - 14321	5.655
7947 - 14186	6.239
6518 - 14162	7.644
11811 - 15398	3.587
10651 - 15220	4.569
10604 - 14957	4.353
9011 - 14486	5.475
8452 - 15360	6.908
9930 - 16203	6.273
6520 - 11311	4.791
6083 - 13208	7.125

Position:	deletion (Kb):
10bp continued:	
7143 - 16147	9.004
11630 - 16034	4.404
7894 - 13256	5.362
7779 - 13687	5.908
10475 - 14834	4.359
8100 - 14913	6.813
9695 - 13963	4.268
6629 - 12878	6.249
6499 - 13126	6.627
7833 - 12145	4.312
8513 - 14488	5.975
9126 - 15685	6.559
6601 - 10175	3.574
9557 - 13283	3.726
12524 - 16092	3.568
5352 - 13692	8.340
9702 - 13763	4.061
8740 - 15921	7.181
8723 - 12965	4.242
8759 - 13172	4.413
11815 - 15568	3.753
8781 - 14349	5.568
8501 - 14695	6.194
8501 - 15175	6.674
5818 - 11392	5.574
7106 - 13506	6.400
5580 - 11103	5.523
9714 - 13738	4.024
6756 - 11971	5.213
6724 - 14220	7.496
8150 - 14836	6.686
9094 - 12961	3.867
9080 - 15536	6.456
7898 - 11727	3.829
10221 - 13760	3.539
7447 - 11954	4.507
5363 - 14816	9.453
5564 - 11977	6.413
5899 - 11174	5.275
8887 - 13123	4.236
6556 - 10810	4.254
10507 - 16205	5.698
10514 - 16205	5.691
11293 - 16030	4.737

Table 5.1.4. Direct repeats within the mouse mitochondrial genome which are positioned within the ATPase 8 gene and the ND5 gene:

This table displays the only direct repeats within the mouse mitochondrial genome of 10bp or larger, where one of the repeat sequences lies within the ATPase 8 gene, and the other is positioned within the ND5 gene. The nucleotide positions and sequences of these two 10bp direct repeats are listed, and the size of the expected deletion which would result if either of these repeats were to represent deletion 'hot spots' is displayed. In addition, the nucleotide positions and the sequence of the 13bp direct repeat which surrounds the most common deletion within the human genome is also shown. The greatest % homology each of the mouse 10bp repeats shares with any 10 consecutive bases of the 13bp human direct repeat was also calculated.

Repeat size:	Position:		Sequence:	Deletion:	%Homology:
Mouse mtDNA:					
10bp	7894 ATPase 8	13256 ND5	A A C C A T A A A A	5.362Kb	40%
10bp	7833 ATPase 8	12145 ND5	T A T T T C A A C T	4.312Kb	50%
Human mtDNA:					
13bp	8470 ATPase 8	13447 ND5	A C C T C C C T C A C C A	4.977Kb	

described in chapter 3. Hence, if any of these represented potential deletion 'hot spots', any such deletions resulting from these repeats would have been detected by our established PCR scheme.

Summary:-

From analyzing the 480 direct repeat sequences of the mouse mtDNA genome of 10bp or greater, only 2 different direct repeats were found to be positioned within the ATPase 8 and ND5 genes, where the 13bp direct repeat sequences which surround the most common deletion within the human genome are positioned. The greatest percentage homology each of these 10bp direct repeats shared with any 10 consecutive bases of the human 13bp repeat was 50% and 40%. If the location of either of these repeat sequences represented a deletion 'hot spot' within the mouse genome, the deletions generated of 4.312Kb and 5.362Kb would have been detected by the two part PCR method described in chapter 3. The 4.312Kb deletion would have been detected by the primer pairs L567-H1295, L480-H1295, L567-H1452 and L480-H1452, generating deleted PCR products of 2.986Kb, 3.857Kb, 4.558Kb and 5.429Kb respectively, while the 5.362Kb deletion would have been detected by the primer pairs L567-H1452 and L480-H1452, generating deleted PCR products of 3.5Kb and 4.379Kb respectively. However, DNA bands of these sizes were not amplified by any of these primer pair combinations.

Subsequent analysis of all the direct repeats confined to the major region between the two origins of replication failed to reveal any repeats which were positioned within both the ATPase 6 and the ND5 genes, which are the sites where a 16bp repeat within the rat genome has been discovered to surround a 4.8Kb age-related deletion. However, the majority of these repeats which were positioned at least 3.5Kb apart were bounded by various combinations of the primers used in the previously

developed two part PCR method, hence any deletions arising from these repeats would have been detected.

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

Experimental design:-

As previously mentioned, any deletions arising from the majority of the direct repeat sequences located within the major region between the two origins of replication, which are at least 3.5Kb apart, would have been detected by the previously developed two part PCR method. However, closer analysis of these repeats revealed that there were 27 direct repeats which had one repeat sequence which was positioned outside of the furthest H strand primer, H1452. Thus, if any one of these 27 repeats was responsible for generating a deletion within the mouse genome, the previous primer pair combinations would be ineffective in amplifying a deleted PCR product. Therefore, the different brain mtDNA samples were re-analyzed using a modified PCR method to scan the genome past H1452, which can be seen in Diagram 5.2.

This PCR method involved the addition of another H strand primer at the nucleotide position 79-60. Since this new H6 primer would result in large products having to be amplified when using the original L567 and L480 primers, and bearing in mind that 4.7Kb was the largest product which can be reliably detected, these two L strand primers were omitted, and another L primer at the nucleotide position 8221-8231 was included. Thus, this new modified method involved the following primer pair combinations; L821-H1107, L821-H1295, L821-H1452 and L821-H6, which would generate PCR products of 2.880Kb, 4.762Kb, 6.334Kb and 8.153Kb respectively. These primer pairs would provide adequate coverage of the regions spanned by 23 of

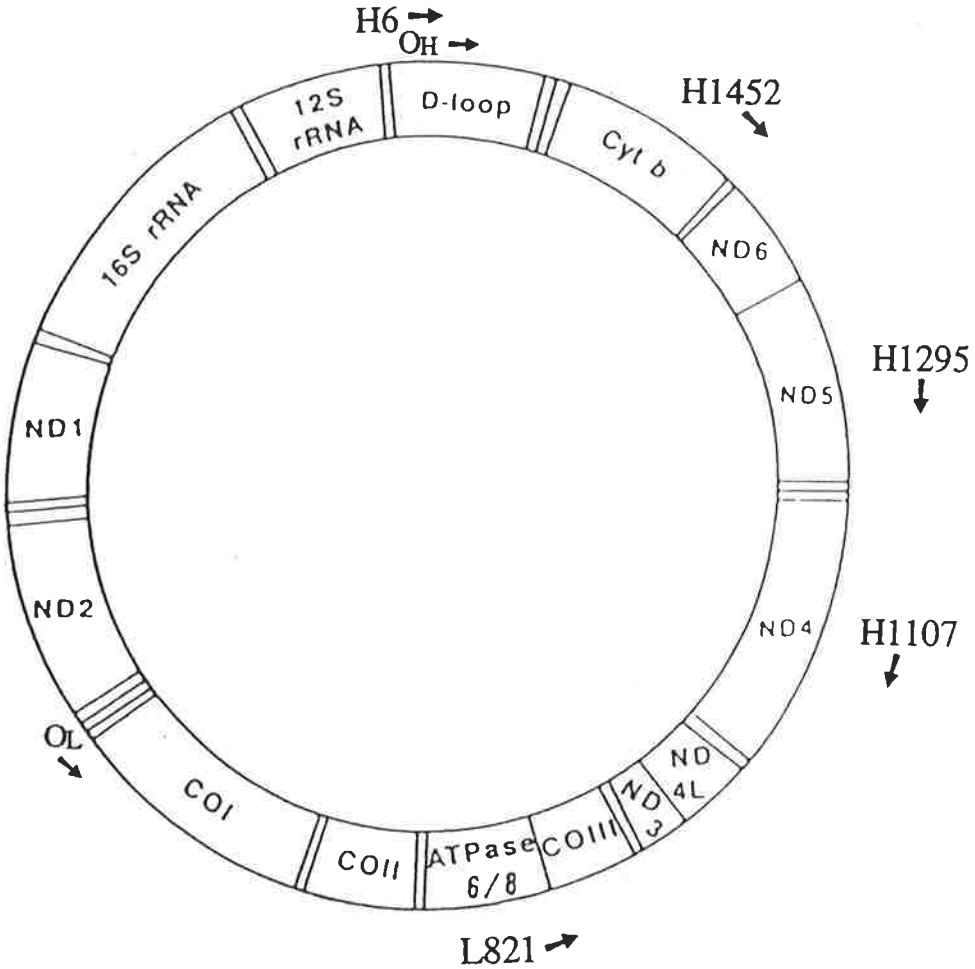


Diagram 5.2. A modified PCR method for scanning the mouse mitochondrial genome for deletions:

This diagram displays the genetic map of the mouse mitochondrial genome, showing the respective positions of the primers used to scan the genome from the nucleotide position 8211 to 79, for the presence of deletions. This PCR method involves the following primer pair combinations; L821-H1107, L821-H1295, L821-H1452 and L821-H6. The open bars represent tRNA (transfer RNA) genes. 12srRNA and 16srRNA are ribosomal RNA genes. ND1, ND2, ND3, ND4L, ND4, ND5 and ND6 genes code for subunits 1, 2, 3, 4L, 4, 5 and 6 of NADH dehydrogenase (Complex 1). COI, COII and COIII genes code for subunits 1, 2 and 3 of Cytochrome c oxidase (Complex IV), the cyt.b gene codes for Cytochrome b, a subunit of Ubiquinol-cytochrome c oxidoreductase (Complex III), and ATPase 6 and 8 genes code for 2 subunits of ATPase Synthase (Complex V). The light and the heavy strand origins of replication are denoted as O_L and O_H respectively.

the 27 repeat sequences which would not have been previously analyzed.

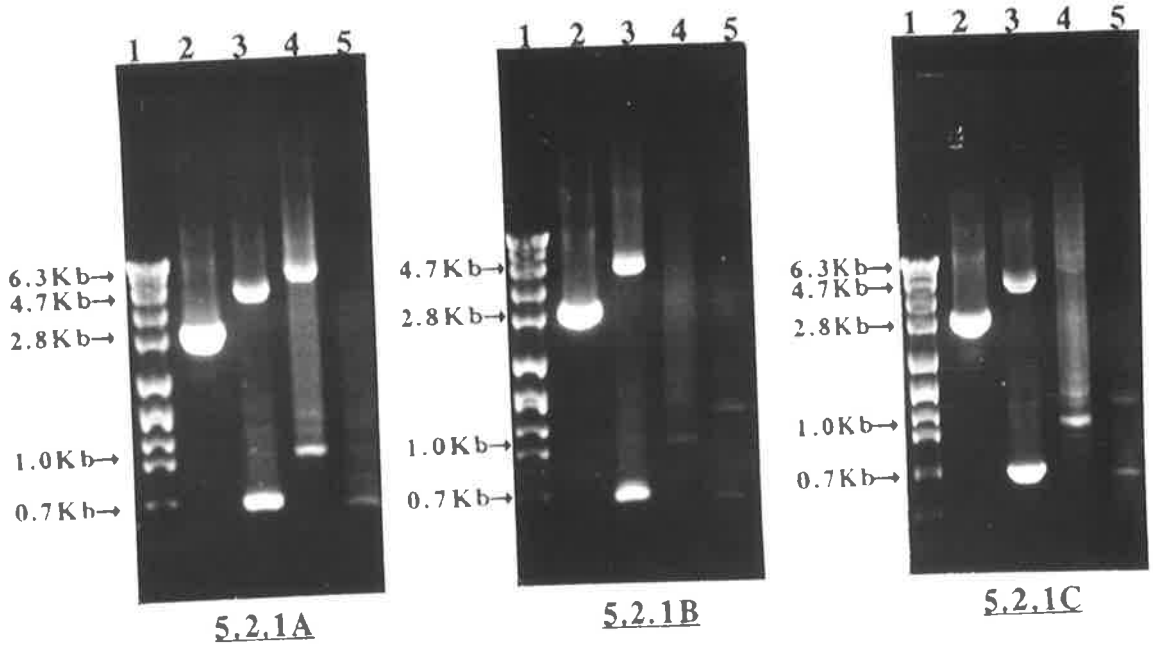
Within any experiment, at least one brain sample from each age group (10 weeks old, 19 months old and 42 months old), was analyzed at the same time, and master mixtures of the reaction reagents were prepared where possible. All the PCR conditions were kept the same as those previously used, with only the primer pair combinations being changed. Following the initial screening by these four primer pairs, the size of the products generated by each primer pair combination were calculated and compared to determine if any of these displayed a corresponding increase in size when the H strand primer was shifted. The banding patterns obtained from each age group were then compared to determine if any of the products were age dependent.

This procedure was repeated using shorter PCR extension times of 40 and subsequently 20 seconds to enhance the generation of smaller deleted PCR products. A further attempt at increasing the amplification of deleted PCR products was also performed by removing an aliquot from the initial 40 cycle PCR reaction of each primer pair combination and diluting it to 1 in 10 000. The diluted samples from each of the four reactions were then added as templates for a second identical 40 cycle PCR reaction. Again, the size of the products amplified by each primer pair combination were calculated and compared to determine if any displayed a corresponding change in size when the H strand primer was shifted.

Results:-

5.2.1. PCR analysis of different aged brain mtDNA samples:

Representative gels showing the results obtained when a 10 week old, a 19 month old and a 42 month old mouse brain mtDNA sample were analyzed by the modified PCR method are shown in Figures 5.2.1A, B & C respectively. With the primer pair L821-H1107, a definite PCR product of 2.8Kb was amplified from each



Figures 5.2.1A, B & C. PCR analysis of different aged brain mtDNA samples by the modified PCR method:
Brain mtDNA from a 10 week old, a 19 month old and a 42 month old mouse were analyzed by the modified PCR method for the presence of deletions (Figure 5.2.1A, B & C respectively). This involved the analysis of each sample by the following primer pair combinations; L821-H1107, L821-H1295, L821-H1452 and L821-H6 (lanes 2, 3, 4 and 5 respectively within each figure). DNA size markers ranging from 8.5Kb to 0.36Kb were run in lane 1 of each figure.

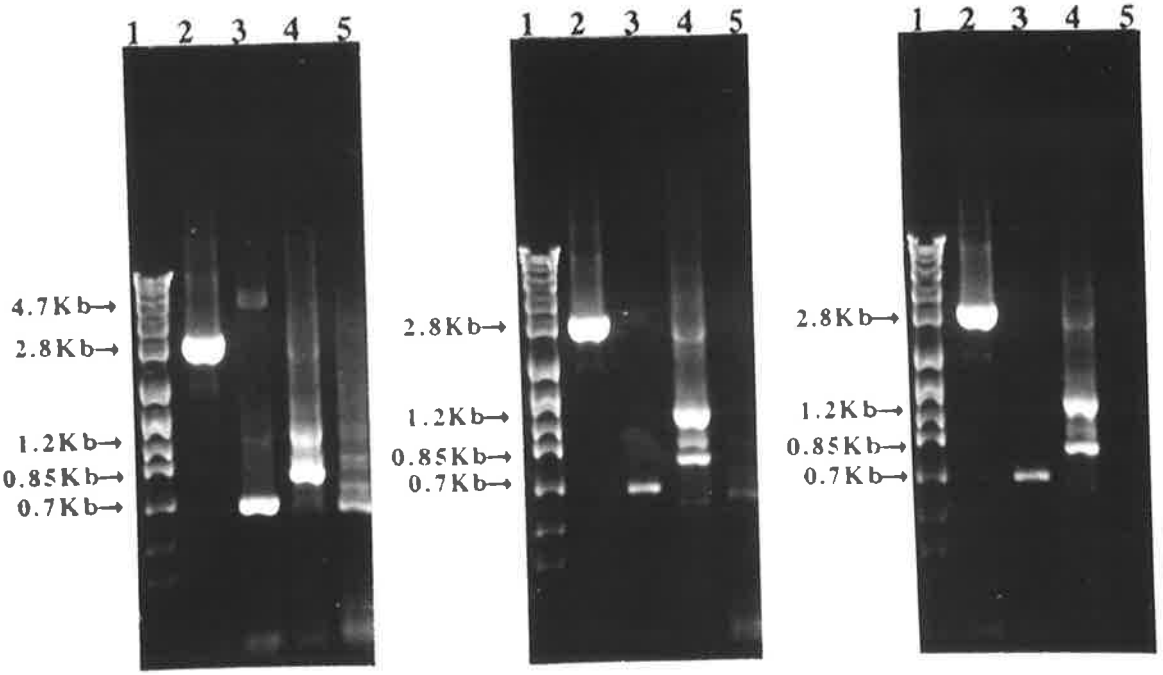
mtDNA sample regardless of age, which corresponds to the expected PCR product which should be synthesized by these primers (lane 2 of each figure). This PCR product showed a corresponding increase in size to 4.7Kb when the H primer was shifted to H1295 (lane 3 of each figure). In addition, a smaller band of approximately 0.7Kb was also observed within this lane for each of the different aged mtDNA samples.

PCR analysis with the primers L821 and H1452 resulted in the synthesis of the expected 6.3Kb PCR product from the 10 week old and the 42 month old samples (Figures 5.2.1A & C, lane 4), in addition to two smaller products of 1.2Kb and 1.0Kb which were also observed within this lane. Analysis of the 19 month old sample by these same primers only resulted in the 1.0Kb product being generated (Figure 5.2.1B, lane 4). Changing the position of the H primer to H6 resulted in two products of 1.2Kb and 0.7Kb being amplified from the 19 month old and the 42 month old samples (Figures 5.2.1B & C, lane 5), while analysis of the 10 week old sample resulted in a 0.7Kb band and three larger very faint bands of approximately 0.8Kb, 1.0Kb and 1.2Kb being observed (Figure 5.2.1A, lane 5).

Overall, the only band which displayed a H strand primer shift pattern was the expected PCR product of 2.8Kb produced by the primers L821 and H1107 (lane 2), which showed a corresponding increase in size to 4.7Kb and 6.3Kb when the H primer was shifted to H1295 and H1452 respectively (lanes 3 & 4 respectively).

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

Representative gels showing the results obtained when a 10 week old, a 19 month old and a 42 month old brain mtDNA sample were analyzed by the modified PCR method, using a reduced PCR extension time of 40 seconds can be seen in Figures 5.2.2A, B & C respectively. As can be seen in lane 2 of each figure, reducing the extension time to 40 seconds still enabled the expected PCR product of 2.8Kb to be



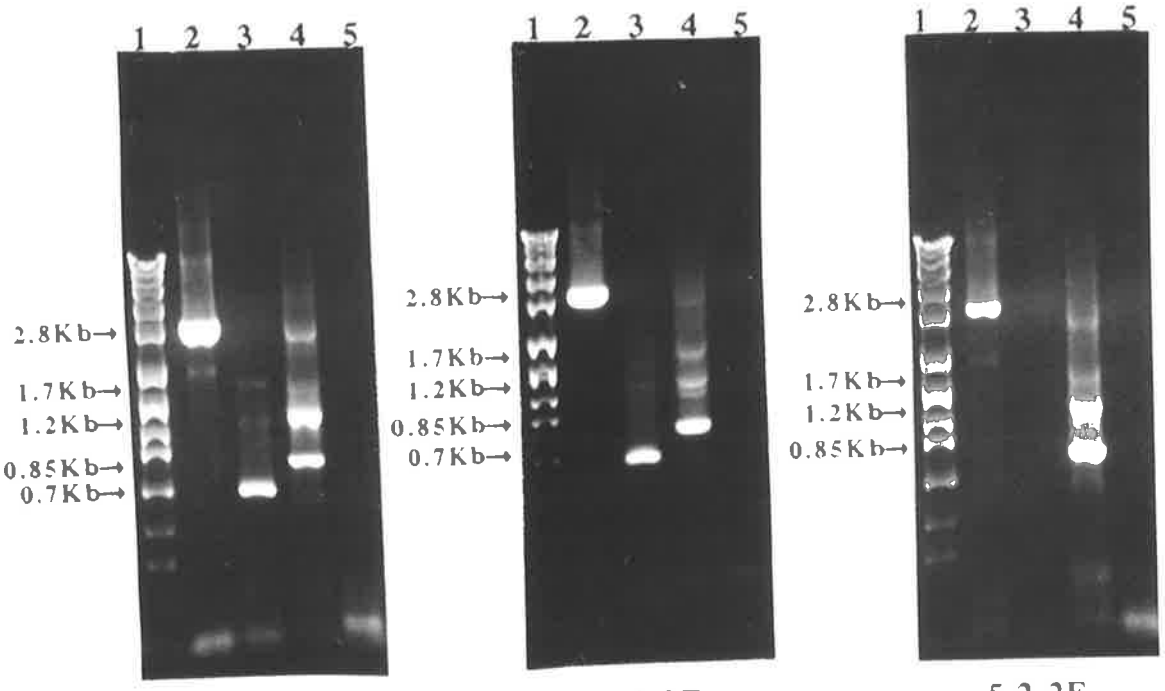
Figures 5.2.2A, B & C. PCR analysis of brain mtDNA by the modified PCR method using a reduced PCR extension time of 40 seconds:
Brain mtDNA from a 10 week old, a 19 month old and a 42 month old mouse were analyzed by the modified PCR method using a reduced PCR extension time of 40 seconds (Figure 5.2.2A, B & C respectively). This involved the analysis of each sample by the following primer pair combinations; L821-H1107, L821-H1295, L821-H1452 and L821-H6 (lanes 2, 3, 4 and 5 respectively of each figure). DNA size markers ranging from 8.5Kb to 0.36 Kb were run in lane 1 of each figure.

amplified by the primers L821 and H1107. In addition to this expected product, a very faint band of approximately 2.0Kb was also observed within each figure regardless of age. Shifting the H primer to H1295 resulted in the expected 4.7Kb PCR product and a smaller, faint band of 1.1Kb being synthesized from the 10 week old sample (Figure 5.2.2A, lane 3), while the previously observed 0.7Kb product was still generated from each DNA sample (lane 3 of each figure).

PCR analysis with the primer pair L821-H1452 resulted in four products being synthesized from each DNA sample with sizes of 2.75Kb, 1.2Kb, 1.0Kb and 0.85Kb (lane 4 of each figure). These bands did not show a corresponding increase in size when the H primer was shifted to H6. With this primer pair H821-H6, four products of 1.2Kb, 1.0Kb, 0.8Kb and 0.7Kb were amplified from the 10 week old sample (Figure 5.2.2A, lane 5), while only a 0.7Kb product was synthesized from the 19 month old sample (Figure 5.2.2B, lane 5). No bands were observed following the analysis of the 42 month old sample by these primers (Figure 5.2.2C, lane 5).

Overall, the only band which displayed a H strand primer shift pattern was the expected PCR product generated by the primers L821 and H1107 of 2.8Kb (lane 2), which displayed a corresponding increase in size to 4.7Kb when the H primer was shifted to the nucleotide position 12954 (lane 3).

The results obtained when the extension time was further reduced to only 20 seconds can be seen in the Figures 5.2.2D, E & F, which correspond to the analysis of a 10 week old, a 19 month old and a 42 month old mtDNA sample respectively. Again the expected PCR product of 2.8Kb, generated by the primers L821 and H1107, was synthesized from each DNA sample regardless of age, however the intensity of this 2.8Kb band was reduced (lane 2 of each figure). Shifting the H primer to H1295, again resulted in a 0.7Kb product being generated from the 10 week old and the 19 month old samples (Figures 5.2.2D & E, lane 3), while no bands were observed following the analysis of the 42 month old sample (Figure 5.2.2F, lane 3). In addition, a very faint band of approximately 1.7Kb was also observed within this lane following the analysis of the 10 week old sample (Figure 5.2.2D, lane 3).



Figures 5.2.2D, E & F. PCR analysis of brain mtDNA by the modified PCR method using a reduced PCR extension time of 20 seconds:
Brain mtDNA from a 10 week old, a 19 month old and a 42 month old mouse were analyzed by the modified PCR method using a reduced PCR extension time of 20 seconds (Figure 5.2.2D, E & F respectively). This involved analysis of each sample by the following primer pair combinations; L821-H1107, L821-H1295, L821-H1452 and L821-H6 (lanes 2, 3, 4 and 5 respectively). DNA size markers ranging from 8.5Kb to 0.36 Kb were run in lane 1 of each figure.

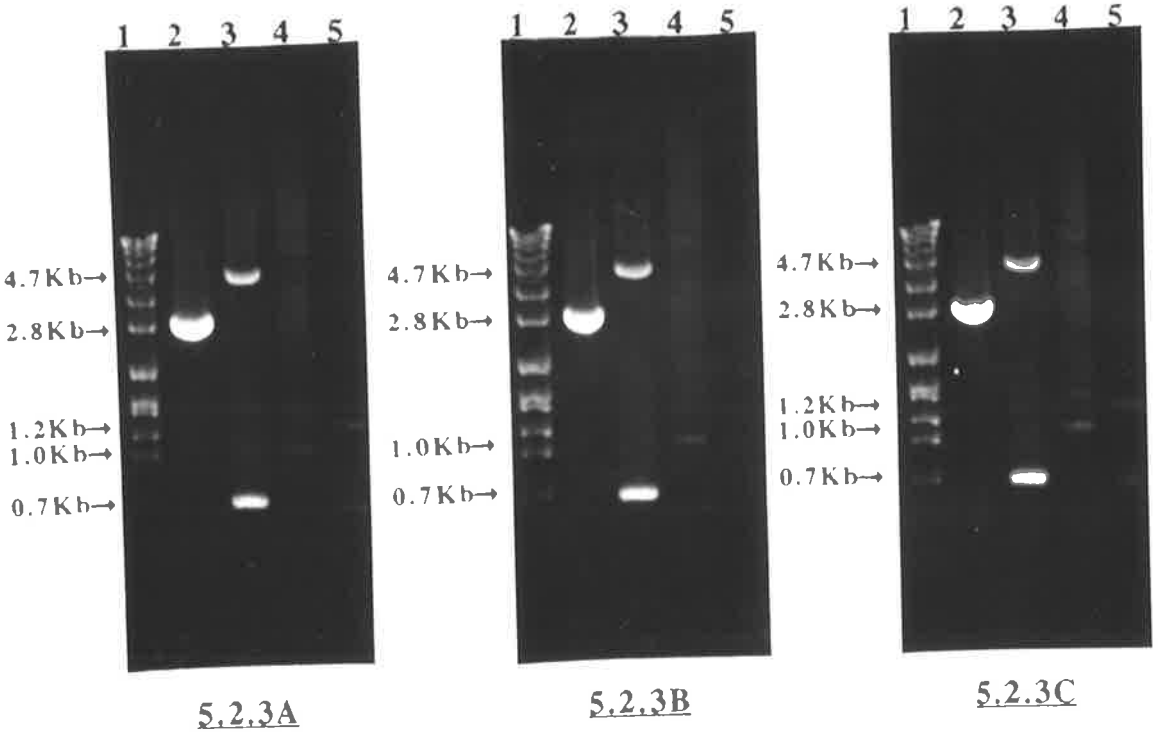
PCR analysis by the primer pair L821-H1452 resulted in four products being amplified from each sample, which had sizes of 2.7Kb, 1.7Kb, 1.2Kb and 0.85Kb (lane 4 of each figure), and shifting the H primer to H6, resulted in no products being synthesized from any of the DNA samples (lane 5 of each figure). Therefore, overall none of the bands observed within any of these three gels displayed a H strand primer shift pattern.

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

Figures 5.2.3A, B & C represent the results obtained when a 10 week old, a 19 month old and a 42 month old mouse brain mtDNA sample respectively, were analyzed by each primer pair within the modified PCR method for a total of 80 cycles. The expected PCR product of 2.8Kb, generated by the primers L821 and H1107, was amplified from each of the different DNA samples regardless of age, as can be seen in lane 2 of each figure. This product displayed a corresponding increase in size to 4.7Kb when the H primer was shifted to H1295 (lane 3 of each figure). In addition, the 0.7Kb product was still generated from each of the DNA samples with this primer pair L821-H1295.

PCR analysis with the primers L821-H1452 resulted in an extremely faint band of approximately 1.0Kb being observed within each gel (lane 4 of each figure), and subsequently shifting the H primer to H6 resulted in two products of 1.2Kb and 0.7Kb being generated from the 10 week old and the 42 month old samples (Figures 5.2.3A & C, lane 5), while no products were amplified during the analysis of the 19 month old sample by these primers (Figure 5.2.3B, lane 5).

Overall, the only band which displayed a H strand primer shift pattern was the 2.8Kb, expected PCR product generated by the primer pair L821-H1107 (lane 2 of each figure), which showed a corresponding increase in size to 4.7Kb when the H primer was shifted to H1295 (lane 3 of each figure).



Figures 5.2.3A, B & C. PCR analysis of various aged brain mtDNA samples for a total of 80 PCR cycles:
Brain mtDNA from a 10 week old, a 19 month old and a 42 month old mouse were analyzed by the modified PCR method for a total of 80 PCR cycles (Figure 5.2.3A, B & C respectively). This involved the analysis of each sample by the following primer pair combinations; L821-H1107, L821-H1295, L821-H1452 and L821-H6 (lanes 2, 3, 4 and 5 respectively in each figure). DNA size markers ranging from 8.5Kb to 0.36Kb were run in lane 1 of each figure.

Summary:-

Re-analyzing the different aged brain mtDNA samples by the modified PCR method, which involved the primer pairs L821-H1107, L821-H1295, L821-H1452 and L821-H6, resulted in no age dependent, deleted PCR products being amplified, which displayed a primer shift pattern. This suggests that the DNA products generated by each different primer pair combination are most likely the result of one or both of the primers misannealing to the wildtype DNA template. Even reducing the PCR extension time, and increasing the number of PCR cycles did not enhance the production of any possible age dependent deleted PCR products, showing a H strand primer shift pattern.

5.3. Conclusions:-

Computer analysis of the mouse mitochondrial DNA genome revealed 480 different direct repeat sequences ranging from 10-16bp in size. 245 of these were confined to the major region between the two origins of replication, while 55 direct repeats were located within the minor area, and 180 of these repeats had one repeat sequence located within the major region and the other sequence positioned within the minor region. Only two of the 245 repeats confined to the major region were found to share the same gene locations as the 13bp direct repeat which surrounds the most common 4.977Kb, human mtDNA deletion. The sequences of these two 10bp repeats were found to share only 50 and 40% homology with any 10 consecutive bases of the human 13bp direct repeat. In contrast, it was not possible to map two mouse direct repeats which displayed exactly the same gene locations as the 16bp repeat which has been found to surround a 4.8Kb deletion within the ageing rat.

Following the analysis of all the repeats within the major region which would theoretically have the potential to generate deletions of 3.5Kb or greater, it was found that the majority of these repeats were positioned within the confines of the primers

used within the previously developed two part PCR method. Thus, if any of these represented deletion 'hot spots' within the mouse mitochondrial genome, these deletions would have been detected by the two part PCR scheme. However, 27 of these repeats had one repeat sequence which was positioned outside of the final H primer of this PCR method, hence the different aged brain mtDNA samples were subsequently re-analyzed by a modification of this previously developed PCR method, to scan regions of the mitochondrial genome which included these direct repeats which escaped analysis by the original PCR method. These experiments however, failed to synthesize any age related deleted PCR products. Even decreasing the extension time and increasing the number of PCR cycles did not enhance the amplification of any deleted PCR products.

Chapter 6:

General Discussion

6.0. Introduction:-

The concept that deletions exist within the mitochondrial genome of humans was first recognized by Holt and co-workers in 1988, who discovered that mtDNA molecules bearing deletions of up to 7.0Kb were present within mtDNA samples obtained from patients with mitochondrial myopathies. Whilst they and other researchers continued to investigate this observation, it wasn't until 1990 that the connection between ageing and the accumulation of these deleted genomes was published by Ikebe and co-workers, who discovered that deleted mtDNA molecules existed within the brains of normal aged humans. Over the past four years since this initial investigation, considerable interest has been generated concerning the relationship between ageing and the accumulation of deleted mitochondrial DNA molecules. Numerous reports have now shown that multiple, different sized deletions accumulate not only within the brain mitochondrial DNA, but also within various other aged tissues (Zhang et al 1992, Linnane et al 1990).

Prior to commencing this project, research within this area was focused upon investigating the presence of deletions within the ageing human mitochondrial genome. There was no reference within the literature concerning the appearance of any such deletions accumulating within the ageing mitochondrial genomes of other species, except for a study by Piko et al (1988), which suggested that deletions and/or insertions may exist within the aged mouse genome, since duplex molecules with single-stranded loops were formed when aged mtDNA samples were heat denatured and subsequently reannealed by cooling. Therefore, it was our aim to determine if the accumulation of

deleted mtDNA molecules during the normal ageing process was confined to the human, or whether it was a phenomenon shared by other species.

We selected the mouse as an experimental model due to the fact that the mouse mitochondrial genome has been completely sequenced and is almost identical to the human genome in size and gene content, and there is a high degree of homology between the sequences of these two species (Bibb et al 1981, Anderson et al 1981). Also, since the lifespan of the mouse is relatively shorter than most other mammalian species, this would enable older animals to be more easily obtained. Analysis of the ageing mouse mtDNA would also confirm whether the heteroduplexes observed by Piko et al (1988) were infact due to the presence of deletions within the mitochondrial genome.

The brain, heart, skeletal muscle and liver were selected for analysis since these tissues are rich in non-dividing cells and have repeatedly been shown to accumulate deleted mtDNA with age at a higher rate than other more easily obtained dividing cell populations such as those found in blood (Ikebe et al 1990, Hattori et al 1991b, Katayama et al 1991, and Yen et al 1991, 1992). In order to detect if any age-related deleted mtDNA molecules had accumulated within these tissues, initially a PCR method specific for the mouse mitochondrial genome was developed. Such a sensitive assay was required since the level of deleted molecules which have been found within the aged human tissues, have been too low to be detected by conventional electrophoresis / Southern blotting techniques, and it was anticipated that the levels within the mouse would be similar to those reported within the human studies.

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

Using 6 specifically designed mouse PCR primers, a two part PCR method was developed, which involved using multiple primer pair combinations to methodically

scan the major region between the two origins of replication for deletions. This method was based upon the principles of the primer shift method devised by Sato et al (1989), to confirm if any of the bands generated by each primer pair, were genuine deleted PCR products or abnormal fragments resulting from the primers misannealing to unexpected positions of the genome. Within the previously reported human studies, primer shift PCR methods were also employed, however the majority of these focused upon the most common deletion of 4.977Kb, and to a lesser extent the 7.436Kb deletion and they did not explore the possibility that other different sized deletions may also exist within the human genome.

Zhang and co-workers (1992) were the only group that looked extensively beyond these most common deletions by using various primer pair combinations, and they found that multiple, different sized deleted mtDNA molecules existed within aged human tissues. Therefore, the study described in this thesis also scanned the genome beyond the area between the ATPase 8 gene and the ND5 gene where the most common 4.977Kb deletion within the human has been characterized. Primers were positioned so that the entire major region between the two origins of replication could be examined. In theory, this two part PCR method should be capable of detecting any deletions within the major area between the replication origins provided that the deletion endpoints are confined to the boundaries of at least one primer pair, and the resulting deleted PCR product is less than or equal to 5Kb, since this was the largest product which could be successfully amplified following optimization of the PCR conditions.

The ability of this PCR method to amplify deleted PCR products from heteroplasmic samples containing deleted mitochondrial DNA templates, was verified by analyzing genetically engineered heteroplasmic samples which contained both the wildtype genome and 4.4Kb-deleted mtDNA molecules. Both parts of designed PCR method were found to be successful in amplifying the expected deleted PCR products which displayed both H and L strand primer shift patterns. By serially reducing the amount of deleted mtDNA within this heteroplasmic sample, whilst keeping the total amount of DNA in the reaction constant, the lowest percentage of deleted mtDNA

molecules which still resulted in a visible deleted PCR product being amplified by our PCR method was approximately 0.01%. This would imply that this method was capable of detecting the presence of 1 deleted mtDNA molecule amongst every 10 000 wildtype genomes.

Within the literature, various methods have been used to quantitate the percentage of deleted mtDNA molecules in aged tissues (Cortopassi & Arnheim 1990, Ozawa et al 1990a, Cooper et al 1992, Mann et al 1992, Simonetti et al 1992, Soong et al 1992, Corral-Debrinski et al 1991,1992a, Hayakawa et al 1993, Kitagawa et al 1993, Blanchard et al 1993). The values found using these methods have varied between samples from the same tissue and between samples from different tissues. However, approximately 85% of these studies found levels of deleted mtDNA molecules which were greater than or equal to 0.01%, and in one case the level of molecules harboring a 4.977Kb deletion was calculated to be as high as 12% in adult brain tissue (Corral-Debrinski et al 1992a). Therefore, if the same relative levels of these deleted genomes are present within the ageing mice tissues, our PCR method should have been capable of successfully amplifying visible deleted PCR products from them.

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

Using the designed two part PCR method, overall no age dependent or tissue specific deleted PCR products were amplified from the mtDNA samples obtained from the various aged mice within this study. In total, the same size products were generated by each primer pair regardless of the age or the tissue examined. None of these products were synthesized within the negative controls where the DNA template was omitted, nor were they present within the DNA samples prior to analysis, which indicates that they were generated during the PCR process. These DNA products also failed to display a corresponding shift in size when either the L or the H strand primers

were independently altered, which suggests that these observed bands were not genuine PCR products but were instead most likely the result one or both of the primers misannealing to the wildtype mtDNA template. The likelihood that these non-specific products were the result of primer dimer formation is not favoured, since the sequences of any two of the designed primers were found to contain no complementary regions which might cause them to anneal to each other.

To enhance the synthesis of smaller deleted PCR products, the brain samples were re-analyzed using shorter PCR extension times. This reduction in the PCR extension time has been used by other groups to allow more efficient amplification of the shorter and rarer deleted mtDNA molecules (Cortopassi & Arnheim 1990, Yen et al 1991 & 1992). However, our experiments gave a negative result. It was not possible to detect any DNA bands which were specific to the older age groups and/or showed a primer shift pattern using this approach.

It was thought that maybe the reason why initial attempts had failed to detect deleted mtDNA molecules was because the levels were too low to be significantly amplified by this PCR method. Therefore, the brain mtDNA samples were re-amplified for a total of 80 cycles. It has previously been shown by Ozawa et al (1990a), that the smaller and rarer PCR products are preferentially amplified in the later cycles of the PCR reaction, and Linnane et al (1990) found that by increasing the number of cycles to 60, they could detect the presence of deleted mtDNA molecules in infant samples which were previously not detected when the PCR experiments were only carried out for 30 cycles. However, within our experiments where a total of 80 cycles was performed, again a negative result was obtained where no age dependent, primer shift PCR patterns were observed.

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

As previously mentioned, the majority of the deletions discovered within the human genome have been found to be surrounded by two tandem direct repeat sequences. Therefore, in an effort to predict the location of deletions with the ageing mouse, tandem direct repeat sequences within the mouse mitochondrial genome were analyzed. The search was limited to those repeats which were 10bp or greater since larger repeats of 13bp and 16bp have been found to surround the most common deletions within the human and rat genomes respectively (Cortopassi & Arnheim 1990, Linnane et al 1990, Gadaleta et al 1992). Analysis of all the repeats within the mouse genome of 10bp or greater revealed two repeats which were localized to the same gene positions as the human 13bp direct repeat. These two repeat sequences however, did not share a high degree of homology with the human repeat sequence. It was also interesting to note that no repeats of 10bp or greater within the mouse genome mapped in the same gene positions as two 16bp repeats which have been found to house a 4.8Kb age-related deletion within the rat genome (Gadaleta et al 1992). These observations indicate that the direct repeat sequences which are involved in generating deletions within mtDNA are not conserved among vertebrate species. In addition, Gadaleta et al (1992) could not find any repeat sequences within the rat genome which shared the same gene locations as the most common human 13bp repeat, and Lee et al (1993) discovered age associated deletions within the Rhesus monkey which were located at different gene positions to the human deletions. Hence, these studies highlight the fact that the formation of deletions within the mitochondrial genome is species specific.

From analyzing all the direct repeats within the mouse mitochondrial genome of 10bp or greater it was found that the majority of these repeats were confined to the major region between the origins of replication and were surrounded by various combinations of the primers used within the two part PCR method described previously. Therefore, if any of these represented potential deletion 'hot spots' within the mouse genome, deleted PCR products should have been amplified. However, there

were also several repeats (~11%) which were confined to the major region but were not surrounded by any primer pair combinations. Thus, deletions arising from these repeats would not have been detected by the two part PCR method used. Changing the primers to incorporate the regions spanned by these direct repeats however failed to generate any age dependent products displaying a primer shift pattern. Again decreasing the extension time and increasing the number of PCR amplification cycles did not enhance the production of any age-related deleted PCR products.

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

There are a number of possible explanations as to why deleted mtDNA molecules were not detected within the tissues of those mice which were analyzed within this study. These can be divided into two categories. Firstly, assuming that deleted molecules were present within the samples analyzed, there exists the possibility that the two part PCR method was not optimal for detecting these deleted molecules and secondly, the possibility that the level of deleted mtDNA molecules was undetectable within the tissues of those mice which were examined, cannot be excluded.

Following the assumption that these various aged mice tissues were heteroplasmic, there are a number of possible reasons why deleted PCR products were not amplified, which may include one or more of the following: (i) Firstly, in contrast to the relatively large deletions which have been discovered within the ageing human genome, smaller size deletions may accumulate within the mouse genome, which would result in larger deleted PCR products having to be synthesized. Since 5Kb was the largest PCR product which could be synthesized within our PCR system, deletions between any two primers which resulted in a deleted PCR product of greater than 5Kb having to be amplified, would not have been detected. For example, if a 3.5Kb or smaller deletion existed between the primers L567 and H1452, which also removed the

H1295 primer binding site, this would in theory result in deleted PCR products ranging from 5.4Kb to 8.8Kb having to be synthesized by this primer pair, L567-H1452. Due to their large size, these deleted products would not have been successfully amplified, and consequently, these deleted mtDNA molecules would have remained undetected. Thus, our PCR assay relied on relatively large deletions to bring any two primers close enough together to efficiently produce a product of 5Kb or smaller.

(ii) Secondly, in order to generate a deleted mtDNA PCR product, the primers have to surround the endpoints of the deletion. Therefore, if a deletion causes the removal of a particular primer binding site, then obviously this primer is no longer useful in detecting the deletion. Due to the developed two part PCR method involving multiple primer pair combinations, the removal of one or more PCR primer binding sites would not pose a problem since the remaining primers would be able to generate deleted PCR products. However, there are a number of situations where deleted PCR products would not be amplified which include:

1) If all the primer binding sites of all the PCR primers were deleted, then obviously no PCR products would be able to be generated. The removal of all of these binding sites could be due to one large deletion, or it is possible that multiple deletions exist within the same mtDNA molecule, which may result in all or numerous combinations of the PCR primer binding sites being removed, such that there is insufficient primer binding sites available to allow any two primers to bind and successfully generate a PCR product.

2) If a deletion removed both the L strand primers L567 and L480, no deleted PCR product could be synthesized. Now it is unlikely that molecules would accumulate where both of the primer binding sites corresponding to the L strand primers would be deleted from the genome, since these sites surround the origin of replication of the light strand of the genome, and hence any molecule bearing such a deletion would be incapable of successfully replicating itself. However, a 10.4Kb deletion has been found in the ageing human heart by Corral-Debrinski et al (1992b), which spans the light strand origin of replication. It therefore seems possible that these deletions could exist

within the mouse genome, and they would have remained undetected by our PCR scheme.

3) Finally, if a deletion spanned the genome such that the final H primer, H1452 binding site was removed, this deleted segment would not be surrounded by any of the primer pair combinations utilized within the two part PCR method and hence no deleted PCR products would be amplified.

The removal of specific primer binding sites may also have inhibited the ability of the modified PCR method used in chapter 5 to detect deleted mtDNA molecules. Reasons for this would include: 1) If all the primer binding sites were deleted, then obviously no PCR products could be synthesized; 2) If a deletion spanned the genome such that the annealing site of the final H primer (H6) was removed, successful amplification of the genome could not be performed since there are no further H strand primers to surround this deleted region. Molecules bearing such a deletion would also not be expected to accumulate with age, since the position of this deletion would also remove the origin of replication of the heavy strand of the genome, and so this molecule would not be capable of successfully replicating itself.

(3) Finally, a deletion which removed the L821 primer binding site would prevent the amplification of any deleted PCR products by this modified PCR method. But, if this deletion spanned the genome such that the binding sites for H1107, H1295 and / or H1452 were still present, then deleted PCR products would have been generated by the previous two part PCR method. However, if this deletion was large enough to delete the region between and including both the L821 and the H1452 primer binding sites then this deletion would not have been detected by any of the primer pair combinations used within any of the PCR experiments within this entire study.

Further evidence to support the concept that deletions may exist within this region of the genome comes from analyzing the position of the direct repeat sequences which are localized to the major region between the origins of replication within this mouse genome. It was found that whilst the majority of these repeats of 10bp or greater were surrounded by various primer pair combinations of either the two part PCR

method or the modified PCR method, there were four of these (one 12bp and three 10bp direct repeats), which would have the potential to delete both the L821 and the H1452 primer binding sites. Hence if these direct repeats represented deletion 'hot spots' within the mouse mitochondrial genome, these deletions would not have been surrounded by any of the primer pairs used within either the two part PCR method or in the modified PCR method used in chapter 5, and therefore they would have remained undetected. However, preliminary studies (results not shown) using the primer pairs L567-H6 and L480-H6, which surround these potential deletion regions, have failed to generate any deleted PCR products.

It is also acknowledged that the removal of various primer binding sites may have inhibited any primer shift patterns from being observed. However, despite the absence of any bands displaying a primer shift, the same size and number of products were generated from mtDNA samples within each different age group, which does not favour an age dependent effect. Also, the intensity of the bands synthesized by each primer pair were similar regardless of the age of the sample, and therefore if any of these represented deleted PCR products, this would imply that the same level of deleted mtDNA molecules existed within samples from both the young and the old animals, which is contradictory to the results which have been found within the human ageing studies. To confirm if any of these bands were deleted PCR products, each of the bands would need to be sequenced and mapped within the mouse mtDNA genome.

(iii) Thirdly, in contrast to the human studies where deleted PCR products were detected within agarose gels after 20-30 PCR cycles, deleted mtDNA molecules in the mouse may exist at much lower levels so that even after 40 cycles, insufficient amplification has occurred for a product to be visualized on an ethidium bromide stained gel. This would seem unlikely since after 40 cycles we were able to detect genetically engineered deletions at levels of 0.01% which is comparable with the levels found in the human ageing studies, and further increasing the number of cycles to 80 cycles would have enhanced this detection limit. However, while the possibility exists that deleted molecules were present at such extremely low levels, this would question

the likelihood that these mutations make any significant contribution to the ageing process within these mice.

(iv) Fourthly, mice may have a cellular mechanism by which they successfully remove deleted mtDNA molecules from their mitochondrial gene pool, or they may have a way of selectively inhibiting the replication of these shorter and rarer genomes, so that they do not accumulate with age.

(v) Finally, the possibility that deletions were present within the genomes of these mice which were examined cannot be excluded, since only the major region between the origins of replication was analyzed within this study. Deletions may have been present within other areas of the genome such as in the minor region between the origins of replication. This is supported by the fact that a 3610bp deletion within this minor region has been found in skeletal muscle mtDNA samples obtained from older aged humans (Katayama et al 1991). The likelihood that mtDNA molecules existed which contained deletions which remove one or both of the origins of replication is also possible however, these molecules would not be capable of being replicated and hence they would accumulate at a much slower rate which would be dependent on how frequently this mutational event takes place. Therefore, it is possible that within the ageing mouse, deletions are preferentially formed within other areas of the genome which were not examined within this study.

So far, the possibility that the developed PCR technique was not optimal for detecting the presence of deletions within the ageing mouse genome has been discussed. An alternative explanation for these results is the absence of deleted mtDNA molecules present within the tissues of those mice which were examined within this study. Suggestions why these mice do not contain appreciable levels of deleted mtDNA molecules include the following;

(i) Firstly, as previously mentioned, only the two younger age groups of mice of 10 weeks old and 19 months old were supplied food *ad libitum*, while the 42 month old mice were obtained from a previous dietary restriction study. It has previously been shown that dietary restriction retards the ageing process (Yu et al 1985) and delays the

onset of age-related neoplastic diseases (Maeda et al 1985). In the last few years evidence has indicated that the effective anti-ageing actions of dietary restriction might be related to its ability to attenuate free radical production (Lee & Yu 1990), and enhance cytosolic defence systems (Laganiere & Yu 1989). Thus, due to the possible involvement of free radicals in the formation of mtDNA deletions (Corral-Debrinski et al 1991, Hayakawa et al 1991b, 1992, 1993, Adachi et al 1993), it is possible that dietary restricting the 42 month old mice prevented any appreciable level of deletions from being formed within the mouse genome, which would explain why no deleted PCR products were generated.

The absence of deletions being identified within the 19 month old mitochondrial genomes might then be attributed to the fact that these mice are still not old enough to have accumulated a level of deleted mtDNA molecules which could be significantly amplified by the PCR technique. The absence of deleted molecules within these 19 month old mice is unexpected since the lifespan of this *Balb C* strain of mice has been reported to be approximately 20-24 months. Thus, it was calculated that the 19 month old mice should be equivalent to old age and so it was anticipated that this age group of mice should have accumulated a significant level of deleted mitochondrial genomes.

(ii) Secondly, despite the animals used within this study, and in contrast to other studies, it may be a general phenomenon that deletions of the mtDNA genome do not occur and / or accumulate within the ageing mouse. This would confirm the study by Nelson and co-workers (1993), published late last year, who also found a negative result when looking for a specific deletion within ageing mice, which has previously been found in two wild mice. A possible explanation why deletions are not formed within the mitochondrial genomes of aged mice tissues could be due to their direct repeats sequences not being located within the right positions to result in the correct conformational changes required for a deletion to be formed by either recombination (Mita et al 1990) or slip-replication (Shoffner et al 1989) which are two proposed mechanisms of how these deletions are formed within the mitochondrial DNA genome.

If the presence of deletions within the human genome and other species such as the monkey and the rat, do offer a significant contribution to the ageing process, the absence of deleted mtDNA molecules within the ageing mice would suggest that these mtDNA mutations do not play a significant role within the ageing process of the mouse, and this would imply that mitochondrial deletions may be an effect rather than a cause of ageing. This would not be the first time in which the ageing process of the mouse has been found to differ from other species. Research on telomeres has shown that in the human these shorten with an increase in age (Lindsey et al 1991), however within the mouse these telomeric regions appear to show no significant shortening with age in normal somatic tissue (Kipling & Cooke 1990).

6.5. Conclusions and future directions:-

Within this study, a PCR method was developed to determine if deleted mtDNA molecules accumulate within the tissues of ageing mice. Primers specific for the mouse mitochondrial genome were positioned so that the major region between the two origins of replication could be analyzed for deletions. Overall, no age dependent or tissue specific deleted PCR products were amplified by any of the primer pair combinations utilized within this study. Therefore, this negative result suggests that mtDNA molecules bearing deletions within this major arc of the genome, do not accumulate within the brain, heart, skeletal muscle and liver of 10 week old and 19 month old *ad libitum* fed mice, nor are they present within the tissues of dietary restricted 42 month old mice.

Ideally, this study should be repeated using 42 month old mice which were *ad libitum* fed to confirm if the absence of deleted mtDNA molecules within this age group was due to dietary restriction. These experiments were not performed within this study because these animals were unavailable for analysis. However, one 42 month old *ad libitum* fed mouse has subsequently been obtained, and analysis of the tissues from

this mouse has revealed identical results (results not shown) to those which were obtained following the analysis of the dietary restricted 42 month old mice. Obviously, this negative result requires further investigation involving a larger number of *ad libitum* fed 42 month old mice.

To overcome the possibility that our primers were not designed within appropriate positions to surround the boundaries of any age associated deletions, different primer positions could also be employed to allow other segments of the genome to be amplified. In particular, primers could be designed to amplify the minor arc of the genome between the replication origins. In addition, recent advances in PCR technology now enable larger PCR products to be successfully amplified of up to 20-40Kb (reviewed by Cohen et al 1994). This would allow larger deleted PCR products to be amplified hence, any size deletion could be detected regardless of how far apart the primers were positioned. This ability to perform long range PCR would also over-come the 5Kb amplification limit which existed within the PCR analysis described in this thesis, and as a result, the previously described two part PCR method could be simplified by only using the primers L567 and L480 in combination with the H6 primer. These primer pair combinations would be capable of scanning the entire major region between the replication origins, where any size deleted PCR products could be amplified regardless of the size of the deletion.

The ability to perform long range PCR over 20Kb, would also enable the entire mitochondrial genome, which is 16.5Kb, to be amplified in one reaction. Therefore, molecules harboring any size deletion, within any region of the genome could be identified by displaying an overall decrease in size. While amplifying the entire genome would not identify the specific location of the deletion, it would determine the total number of different size deleted molecules which were present within any given heteroplasmic sample. The deleted PCR products obtained could then be analyzed by restriction endonuclease digestion and subsequently sequenced to more precisely define the deletion endpoints.

Since the presence of deletions within the ageing human genome were initially identified whilst investigating the presence of deletions within patients with various neuromuscular diseases (Ikebe et al 1990), it is possible that the position of deletions within the ageing mouse could be initially defined by examining mtDNA from mice which have a maternally inherited neurodegenerative disease of mitochondrial origin. To the best of our knowledge prior to commencing this project, there was no mouse neurodegenerative model available for these experiments to be performed. If such a model was obtainable, the level of deleted mtDNA molecules within the mtDNA samples obtained from these diseased mice should be much higher than the levels found within the normally ageing mice, since this is what has been found within the human studies (Corral-Debrinski et al 1992b). As a result, the position of deletions within the mouse mitochondrial genome could be more easily identified prior to examining the genomes of various aged mice. This theory however, relies upon the assumption that the deletions which are formed within the diseased mice are the same as those deletions which accumulate during the normal ageing process of the mouse.

Even though deleted mtDNA molecules were not found within the mouse tissues examined within this study, it does not exclude the possibility that deleted molecules would accumulate in the mouse with age if they were formed within the first place. This could be confirmed by genetically engineering a mouse model which contains deleted mtDNA molecules, and determining whether the proportion of deleted genomes increases with age. Recently, Adachi and coworkers (1993) found that they could induce the formation of deleted mtDNA molecules specifically within the hearts of 4 week old mice by chronic subcutaneous administration of the compound doxorubicin, which stimulates oxidative phosphorylation. Using PCR methodology, they detected a 4Kb deletion which exists within the same region as the majority of deletions which have been identified within the ageing human mitochondrial genome. These mice could be used as an experimental model for studying the age accumulation of deleted mtDNA in the mouse, by ceasing doxorubicin administration after several weeks and determining whether the level of the 4Kb-deleted molecules subsequently

increases with age. However, this compound has been shown to cause chronic cardiac toxicity characterized by congestive heart failure, and so these animals may not be capable of surviving after prolonged exposure to doxorubicin, and as a result they may not be an ideal model for an ageing study.

An alternative approach would be to genetically engineer a mouse model which has a heteroplasmic mitochondrial gene pool at birth. To avoid exposing the entire animal to doxorubicin, cultured cells or isolated mitochondria could be incubated in the presence of doxorubicin for various periods of time. These mitochondria could then be microinjected into embryonic stem (ES) cells. Those ES cells which had accepted mitochondria containing deleted genomes could then be identified by culturing these cells and measuring the level of cytochrome c oxidase (COX) activity, since a decline in COX activity has been found to be associated with the presence of deleted mtDNA molecules (Mita et al 1989,1990, Collins et al 1991, Prelle et al 1994). Those ES cells displaying decreased COX activity could then be introduced into mouse blastocysts, where they would contribute to the developing embryo, and the resulting mice should contain a mixture of deleted and wildtype mtDNA molecules at birth. The presence of the deleted mtDNA molecules within these mice could then be detected by PCR, utilizing the same primers as those used by Adachi et al (1993). As the mice aged, mtDNA from different tissues such as the brain and heart could then be analyzed to determine if the level of these 4Kb-deleted genomes increases with age.

While this study has investigated the relationship between mtDNA deletions and the ageing process within the mouse, it does not explore the possibility that other mutations of the mouse mitochondrial genome may accumulate with age. For example, previous studies have shown that point mutations of the human mitochondrial genome are present in elderly humans (Munscher et al 1993a, 1993b, Zhang et al 1993), and it is therefore possible that point mutations within the mouse genome may also accumulate with age. There are a number of techniques which could be used to analyze the mouse genome for point mutations which include single strand conformation polymorphism analysis (Suomalainen et al 1992b), and denaturing gradient gel

electrophoresis (Yoon et al 1991). Both of these methods have been shown to be capable of detecting mtDNA molecules which differ by a single base pair change.

In addition, other studies could also be performed which indirectly assess the function of the mitochondrial genome. For example, the activity of the respiratory enzyme complexes within the ageing mouse could be analyzed, since numerous reports have shown that especially complexes I and IV, which contain the largest number of mtDNA encoded subunits, decline with age in the human (Trounce et al 1989, Yen et al 1989, Boffoli et al 1994). In particular, the number of human skeletal muscle and heart muscle fibres which display cytochrome oxidase activity (complex IV) have been shown to decrease with age by using histochemical methods (Muller-Hocker et al 1989, 1990, 1992, Byrne et al 1992).

From our experiments, we could not find any deletions within the major region between the origins of replication within the mitochondrial genome of the ageing mouse. While this result is not supportive of the view of 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 are important contributory factors to the ageing process as proposed by Linnane et al (1989).

Appendix:

Bresatec
A.C.N. 007 988 767

SPP-1 BACTERIOPHAGE DNA RESTRICTED WITH *ECO* RI

DESCRIPTION: We find that this DNA provides an excellent range of DNA *Mr* fragments. The *Eco* RI digest of bacteriophage SPP-1 DNA produces 15 fragments of the following sizes[1].

Fragment No	Size (Kb) mean ± SD**	Molecular Weight*
1	8.51 ± 0.07	5.62×10^6
2	7.35 ± 0.06	4.85×10^6
3	6.11 ± 0.06	4.03×10^6
4	4.84 ± 0.03	3.19×10^6
5	3.59 ± 0.01	2.37×10^6
6	2.81 ± 0.01	1.85×10^6
7	1.95 ± 0.01	1.28×10^6
8	1.86 ± 0.02	1.23×10^6
9	1.51 ± 0.01	0.99×10^6
10	1.39 ± 0.02	0.92×10^6
11	1.16 ± 0.01	0.76×10^6
12	0.98 ± 0.01	0.64×10^6
13	0.72 ± 0.00	0.47×10^6
14	0.48 ± 0.01	0.32×10^6
15	0.36 ± 0.01	0.24×10^6

* Assuming *Mr* 660 per base pair
** Standard Deviation was determined from a minimum of 4 tracks.

SPP-1 DNA/*Eco* RI fragments.
0.5 µg/lane. 1% agarose gel stained with ethidium bromide.

Preparation and Use as DNA Molecular Weight Markers: The double-stranded DNA is isolated from bacteriophage SPP-1, which has been propagated in *B. subtilus* (MCB), digested to completion with *Eco* RI restriction enzyme, ethanol precipitated and resuspended in 10 mM Tris-HCl, pH 7.6, 1 mM EDTA. Store at –20°C. Working samples may be kept at 4°C.

The recommended gel loading buffer consists of 5% glycerol, 1 mM EDTA, 0.05% xylene cyanol and 0.05% bromophenol blue. Depending on the size of the gel used, the fractionation of 0.5 µg of this sample should readily allow the detection of all fragments under ultra-violet light after staining with ethidium bromide. Much less DNA may be required if small submarine gels are employed.

For your convenience, an aliquot of 2 x loading buffer is supplied with each shipment.

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