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Human Mitochondrial Genetics

LOIS A. TULLY AND BARBARA C. LEVIN*

Biotechnology Division, National Institute of Standards and Technology, 100 Bureau Drive, Stop 8311, Gaithersburg, MD 20899-8311, U.S.A.

Introduction

The field of human mitochondrial genetics has advanced way beyond where the Human Genome Project hopes to be by the year 2003, the projected year for completing the sequence of the entire human nuclear DNA genome. Not only has the mitochondrial DNA (mtDNA) been completely sequenced, the regions that code for genes and those that are noncoding have been distinguished, the function of all the genes have been determined, the mtDNA genetic code has been shown to differ in some ways from the Universal Genetic Code of nuclear DNA, and a number of diseases have been correlated with specific mitochondrial DNA mutations (http://infinity.gen.emory.edu/mitomap.html).

Some of the current areas of research interest with regard to mitochondrial genetics are: examining the variability among individuals or within a single individual (heteroplasmy); distinguishing between polymorphisms and disease-producing mutations; detecting mutations present in low concentrations in an individual; analysing the effects of chemical or physical agents and the mechanisms which lead

*To whom correspondence may be addressed (barbara.levin@nist.gov)

Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; CO, cytochrome *c* oxidase (sub-units COI, COII, COIII); CPEO, chronic progressive external ophthalmoplegia; CSB, conserved sequence block; Cyt *b*, cytochrome *b*; Cyt *c*, cytochrome *c*; D-loop, displacement loop (also called noncoding or control region); $FADH_2$, flavin adenine dinucleotide, reduced form; HV1, hypervariable region 1; HV2, hypervariable region 2; KSS, Kearns-Sayre syndrome; LHON, Leber's hereditary optic neuropathy; MELAS, mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes; MERRF, myoclonic epilepsy with ragged red fibres; mtDNA, mitochondrial DNA; MtTF1, mitochondrial transcription factor; NADH, nicotinamide adenine dinucleotide, reduced form; NARP, neuropathy, ataxia, retinitis pigmentosa; ND, NADH dehydrogenase (sub-units ND1, ND2, ND3, ND4, ND5, ND6); OXPHOS, oxidative phosphorylation; PCR, polymerase chain reaction; RNase MRP, Mitochondrial RNA processing; RRF, ragged red fibres; TAS, termination-associated sequence.

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to mutations or biochemical defects; understanding the progressive nature of mitochondrial diseases; determining evolutionary relationships among human populations and phylogenetic relationships among species; correlating mitochondrial changes with the ageing process; and understanding the role of mitochondria in the regulation of apoptosis (programmed cell death) (Green and Reed, 1998). All of these areas of scientific inquiry will provide important forensic, medical, phylogenetic or evolutionary information and all of them depend upon the determination of sequence differences.

Several molecular methodologies have been successfully applied to the examination of mitochondrial polymorphisms and mutations. Although sequencing an entire region of DNA provides the most comprehensive genetic information, a number of screening assays have been developed for detecting single base pair changes [eg, single nucleotide polymorphisms (SNPs) and deleterious point mutations], deletions, insertions, and duplications. One of the avenues for providing researchers with necessary controls or calibration tools for use in the analysis of human DNA is the development of Standard Reference Materials (SRMs) by the National Institute of Standards and Technology (NIST). Use of these SRMs can help assure that the data being collected, published, and entered into massive databanks or on the Internet are correct.

This chapter will review some of these current areas of scientific interest with regard to human mitochondrial genetics.

Biogenesis of mitochondria

Mitochondria are commonly believed to have evolved following a symbiotic relationship between early anaerobic eukaryotes, which were unable to metabolize oxygen, and free-living aerobic bacteria, which relied on eukaryotes for a food source. The existence of a DNA genome unique to mitochondria was first reported in 1963 (Nass and Nass, 1963). The mitochondrion is the only cell organelle in the animal kingdom, other than the nucleus, to contain its own DNA genome. Its similarity to bacterial DNA helped stimulate the endosymbiotic theory; these similarities include having circular, double-stranded DNA and protein synthesis which is sensitive to many microbial antibiotics (chloramphenicol, mikamycin, erythromycin, spiramycin and carbomycin) (Mitchell *et al.*, 1975). Recently, however, this endosymbiotic theory has been re-examined in the face of new comparative sequence data (Gray *et al.*, 1999).

Role of mitochondria in cell biochemistry

The mitochondrion is known as the 'powerhouse of the cell' because oxidative phosphorylation (OXPHOS), which produces the ATP needed for cellular energy, occurs in this organelle. The OXPHOS pathway is composed of five multi-enzyme complexes that carry out the electron transport chain culminating in ATP synthesis. In this process, Complex I (NADH dehydrogenase) and Complex II (succinate dehydrogenase) catalyze the oxidation of NADH and $FADH_2$, respectively, by Coenzyme Q. The electrons generated by this process move from Complex III (ubiquinol: cytochrome *c* oxidase), to cytochrome *c* (cyt *c*), then to Complex IV

(cytochrome *c* oxidase), and finally to oxygen, the terminal electron acceptor of the electron transport pathway. Sufficient energy is released by this process to produce a proton gradient that is used by Complex V (ATP synthase) to produce ATP from adenosine diphosphate (ADP), plus inorganic phosphate (P_i). The entire OXPHOS pathway encompasses more than 100 proteins (Shoffner and Wallace, 1995), the majority of which are encoded by nuclear genes. These nuclear-encoded proteins must be transported from the cytoplasm, where they are synthesized, to the mitochondrial inner membrane, where they form complexes with mitochondrial-encoded proteins (Shoffner and Wallace, 1995).

Genetic properties of mitochondrial DNA

Human mtDNA is a circular, double-stranded molecule containing a purine-rich heavy strand (H strand) and a pyrimidine-rich light strand (L strand) (*Figure 6.1*). It was sequenced in its entirety (16,569 base pairs) in 1981 (Anderson *et al.*, 1981) and was shown to consist of a noncoding region (also called the control region or the D-loop), which is about 1,000 base pairs in length, and a coding region. The coding region contains 37 genes, all of which lack introns (Anderson *et al.*, 1981; Wallace, 1992). Twenty-two of the genes encode for transfer RNAs (tRNA), two encode for ribosomal RNAs (12S and 16S), and 13 encode for protein-coding messenger RNAs (mRNA). Twenty-eight of the 37 genes use the H strand as template, including rRNA genes, 14 tRNA genes, and 12 protein genes. The light strand encodes eight tRNA genes and one protein, NADH dehydrogenase 6 (ND6). All of the mitochondrial gene products are used for OXPHOS; however, as stated above, the majority of the proteins needed for OXPHOS are encoded by nuclear genes and transported into the mitochondria.

Most, if not all, scientists sequencing mtDNA since 1981 have used the same numbering system as Anderson (ie, nucleotide positions are numbered from one to 16,569, beginning in the noncoding region and continuing counterclockwise around the circle), and have used the Anderson sequence as a reference with which to compare their results. However, the Anderson sequence is a consensus sequence of mtDNA from a number of sources. The main source was a single human placenta, but they also sequenced some regions of mtDNA from HeLa cells. The results from five regions (bp 10, 934, 935, 14272, and 14365) were ambiguous and were assumed to be the same as that found in bovine mtDNA. Since 1981, many regions of human mtDNA have been sequenced by many laboratories, and the data from that literature now indicates the consensus sequence of Anderson may be incorrect in its designations of approximately 14 base pairs (Howell *et al.*, 1992b; Marzuki *et al.*, 1992). Results at NIST on the mtDNA sequence of three apparently normal individuals agreed with 12 of the 14 proposed discrepancies with Anderson (*Table 6.1*) (Levin *et al.*, 1999).

Inheritance properties of mitochondrial DNA

The inheritance properties of mtDNA are very different from those of nuclear DNA. While there are only two copies of nuclear DNA present in each somatic cell, the cytoplasm contains multiple mitochondria; this number of mitochondria has been

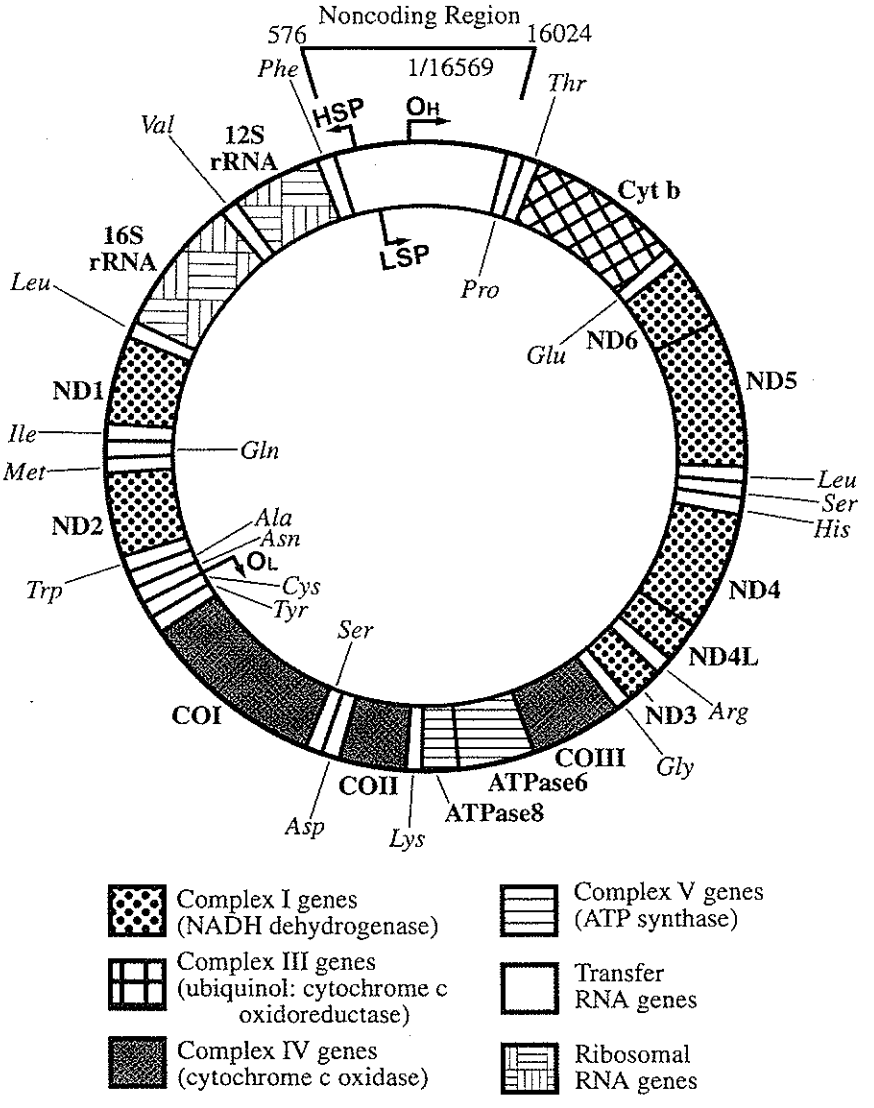

Figure 6.1. The human mitochondrial DNA genome. The structural genes for the sub-units of NADH dehydrogenase (ND1, ND2, ND3, ND4, ND4L, ND5, ND6) cytochrome *c* oxidase (CO I, CO II, CO III), cytochrome *b* (cyt *b*), and ATP synthase (ATPase 6, ATPase 8) are shown, as well as the 12S and 16S ribosomal RNA (rRNA) and 22 transfer RNA (three letter symbols) genes. The origins of replication for the heavy strand (O_H) and light strand (O_L), and the promoters for the heavy strand (HSP) and light strand (LSP) are designated by arrows.

estimated to range from about a dozen to more than 1,000 (Robin and Wong, 1988). Additionally, each mitochondrion can contain more than one DNA molecule. Therefore, the number of mtDNA molecules in somatic cells can be as few as several dozen to as many as several thousand (Bogenhagen and Clayton, 1974; Michaels *et al.*, 1982; Reis and Goldstein, 1983; Robin and Wong, 1988). The greatest numbers are

Table 6.1. Errors vs. polymorphisms in Anderson mtDNA sequence

Basepair No.	Anderson designation → Literature designation (No. found/No. examined)	Anderson designation → NIST designation (No. found/No. examined)	Change
1438	A → G[1]	A → G (3/3)	12S rRNA
3423	G → T (87/87)[2]	G → T (3/3)	SILENT
4769	A → G (28/30)[2]	A → G (3/3)	SILENT
4985	G → A (9/9)[2]	G → A (3/3)	SILENT
8860	A → G[1]	A → G (3/3)	Thr → Ala
11335	T → C (8/8)[2]	T → C (3/3)	SILENT
11719	G → A (26/37)[2]	G → A (1/3)	SILENT
12308	A → G (3/9)[2]	NOT FOUND	$tRNA^{leu}$
13702	G → C (105/105)[2]	G → C (3/3)	Gly → Arg
14199	G → T (9/9)[2]	G → T (3/3)	Pro → Thr
14272	G → C (9/9)[2]	G → C (3/3)	Phe → Leu
14365	G → C (9/9)[2]	G → C (3/3)	SILENT
14368	G → C (9/9)[2]	G → C (3/3)	Phe → Leu
15326	A → G (6/6)[2]	A → G (3/3)	Thr → Ala

[1]Marzuki *et al.*, 1992.
[2]Howell *et al.*, 1992b.

found in the tissues, such as brain, skeletal muscle, and retina, with the highest oxygen demands.

Analysis of mtDNA polymorphisms in several human pedigrees demonstrated that mtDNA is maternally inherited and does not undergo recombination (Giles *et al.*, 1980); recently, however, some investigators have questioned this thesis based on evolutionary population studies (Eyre-Walker *et al.*, 1999; Hagelberg *et al.*, 1999). Maternal inheritance may be partially explained by the small number of mtDNAs found in spermatozoa (approximately 50–100) (Birky, 1983) compared to the number in the oocyte (100,000–200,000) (Michaels *et al.*, 1982; Chen *et al.*, 1995). Although the sperm may introduce a small number of mitochondria into the egg at fertilization, evidence has shown that they are digested shortly after penetration (Smith and Alcivar, 1993). However, using mice, Gyllensten *et al.* (1991) demonstrated the occurrence of paternal inheritance in *inter*specific (ie parents with different nuclear backgrounds) crosses. Through repeated matings of M. *spretus* females to C57BL males, low levels of mtDNA with paternal sequences (0.01–0.1% relative to maternal contributions) were detected in offspring using a sensitive polymerase chain reaction (PCR)-based detection method. These results were in agreement with earlier studies of *inter*specific crosses in drosophila (Kondo *et al.*, 1990).

More recently, however, the implication that paternal inheritance of mtDNA occurs naturally in mammals was disputed by other investigators. When Kaneda *et al.* (1995) studied *inter*specific mouse hybrids, their results were in agreement with Gyllensten *et al.* (1991), and paternally-derived mtDNA sequences were detected in neonates in the F1 generation. However, experiments with *intra*specific mouse hybrids (ie parents had identical nuclear backgrounds but contained mtDNA from different subspecies) were able to detect paternal mtDNA only through the pronucleus stage of development. These findings suggest the existence of species-specific mechanisms that direct the recognition and elimination of sperm-derived mitochondria during early embryogenesis. It has been proposed that the paternally-derived

mtDNA that remained in the offspring of *inters*pecific mouse crosses may have escaped this recognition process because of the different genetic origins (ie species) of the parents (Gyllensten *et al.*, 1991; Kaneda *et al.*, 1995; Manfredi *et al.*, 1997).

The maternal inheritance hypothesis has been further supported by other studies. Sutovsky *et al.* (1996) studied sperm incorporation and conversion of sperm-derived components into zygotic structures during *in vitro* fertilization of bovine oocytes and found that the sperm mitochondria became undetectable in late four-cell stage embryos. Manfredi *et al.* (1997) co-incubated live human sperm with human somatic cells devoid of mtDNA (ρ0 cells), and found that although 10–20% of the cells contained functioning sperm mitochondria immediately following sperm entry, only a very small fraction of the cells ($1/10^5$) survived more than 48 hours. These findings were in contrast to experiments using somatic cell-derived mtDNAs (King and Attardi, 1988) where rapid replacement of the resident mtDNA by the exogenous mtDNA occurred, further supporting the hypothesis that mechanisms exist to specifically eliminate sperm- but not somatic cell-derived mtDNA.

Replication, transcription, and translation

The noncoding region of human mtDNA is 1,122 base pairs in length and is located between the $tRNA^{Phe}$ and $tRNA^{Pro}$ genes, at nucleotide positions 16,024 to 576 (*Figure 6.1*). The origin of replication for the heavy strand (O_H), transcription initiation promoters for both the heavy and light strands, and regulatory sequences for replication and transcription are found in this region (Clayton, 1982, 1991, 1992; Shoffner and Wallace, 1995). Replication begins at O_H and is catalyzed by mtDNA polymerase γ, a nuclear encoded enzyme that is imported into the mitochondria (Schon, 1993). The heavy strand is replicated by the polymerase until it reaches the light strand origin of replication (O_L), located 2/3 of the way around the genome between $tRNA^{Asn}$ and $tRNA^{Cys}$. Synthesis of the light strand then begins in the opposite direction (Clayton, 1982; Lestienne and Bataille, 1994; Shoffner and Wallace, 1995). Replication continues until synthesis is completed on both strands, resulting in a catenated pair of circles. The linked rings are broken and resealed by DNA ligase to produce two daughter duplex mtDNA genomes. This entire process takes about two hours (Schon, 1993).

However, the majority of the replication cycles which initiate at O_H do not result in the synthesis of a full length genome; instead they are terminated at a specific 15 base pair site in the noncoding region called the termination associated sequence (TAS). In humans, this sequence is located at nucleotide positions 16,157–16,172, a short distance from the 3' end of the noncoding region and approximately 500 bases downstream from O_H (Doda *et al.*, 1981; Clayton, 1982; Madsen *et al.*, 1993; Shoffner and Wallace, 1995). This newly synthesized, terminated strand remains annealed to the template forming a triplex (reason why the noncoding region is also called the D-loop). Similar sequences have been reported in other species, including mice (Doda *et al.*, 1981), cows, and pigs (MacKay *et al.*, 1986), suggesting that TASs may function as *cis* (ie sequence-specific) elements involved in the regulation of heavy strand synthesis.

Mitochondrial genes are transcribed from each strand as polycistronic RNA molecules (Clayton, 1991, 1992; Attardi, 1993). Transcription of the light strand

Table 6.2. Universal genetic code and (human mtDNA differences)[1]

5' End	2nd Position				3' End
	T	C	A	G	
T	Phe	Ser	Tyr	Cys	T
	Phe	Ser	Tyr	Cys	C
	Leu	Ser	TER[2]	TER[2] (Trp)	A
	Leu	Ser	TER[2]	Trp	G
C	Leu	Pro	His	Arg	T
	Leu	Pro	His	Arg	C
	Leu	Pro	Gln	Arg	A
	Leu	Pro	Gln	Arg	G
A	Ile (INT)[3]	Thr	Asn	Ser	T
	Ile (INT)[3]	Thr	Asn	Ser	C
	Ile (Met) (INT)[3]	Thr	Lys	Arg (TER)[2]	A
	Met (INT)[3]	Thr	Lys	Arg (TER)[2]	G
G	Val	Ala	Asp	Gly	T
	Val	Ala	Asp	Gly	C
	Val	Ala	Glu	Gly	A
	Val	Ala	Glu	Gly	G

[1]mtDNA differences are in parenthesis.
[2]TER: Termination codon.
[3]INT: Initiation codon.

begins at the light strand promoter and continues through the D-loop to eventually generate ND6 and eight tRNA transcripts. Interestingly, the 5' end of the light strand transcript, which is later cleaved by RNase MRP (mitochondrial RNA processing) between conserved sequence blocks (CSB) II and III, also provides a 200 base pair primer used for initiation of DNA replication. Heavy strand transcription takes place in the opposite direction using the heavy strand promoter, which is actually two closely located initiation sites. The upstream site produces a transcript of both 12S and 16S rRNAs, in addition to $tRNA^{Phe}$ and $tRNA^{Val}$. The downstream site results in the synthesis of a large polycistronic transcript which extends the entire length of the heavy strand and contains all of the other tRNAs and mRNAs normally encoded by this strand. The shorter heavy strand transcript is produced at about 25 times the rate of the full-length transcript, ensuring that sufficient 12S and 16S rRNAs will be available for protein translation (Schon, 1993). The specific transcription factor (mtTF1) controlling this process is encoded by nuclear DNA and is a 25 kDa DNA binding protein required for transcription initiation (Clayton, 1991, 1992). MtTF1 activates transcription by binding as a dimer to the light or heavy strand promoter and unwinding the DNA. This introduces negative supercoils, which increase the efficiency of transcription. The heavy strand promoter is activated following the binding of multiple mtTF1 molecules, whereas the light strand promoter requires just a single molecule.

The release of individual mRNA and tRNA transcripts is made possible by the interspersion of the tRNA genes between the rRNA and protein coding genes. A series of four enzymatic activities result in the cleavage of the polycistronic transcripts on the 5' and 3' ends of the tRNA sequences, releasing the mature rRNA, tRNA, and mRNA transcripts (Attardi, 1993). The four enzymatic activities include RNase P activity (cleaves heavy strand polycistronic transcripts on the 5' side of the tRNA

sequences), 3' endonuclease (cleaves the transcripts on the 3' side of tRNA sequences), poly(A) polymerase (adds a stretch of A residues to the 3' ends of mRNAs and rRNAs), and tRNA nucleotidyl transferase activity (adds CCA to the tRNAs).

Translation of the mRNA transcripts takes place on the mitochondrial ribosomes. The mtDNA genetic code differs from the 'Universal Genetic Code' in several ways (*Table 6.2*). For example, in mtDNA, TGA is a tryptophan codon, whereas in the universal code it is a stop codon. Another example is ATA, which is a methionine codon in the mtDNA genetic code and is an isoleucine codon in the universal code. Furthermore, the nuclear DNA genetic code has 32 tRNAs, whereas the codon-anticodon pairing is simplified in the mitochondrial genetic code, only having 22 tRNAs (Attardi, 1993; Shoffner and Wallace, 1995).

Heteroplasmy and replicative segregation

The mutation rate in mtDNA in warm blooded vertebrates is about ten times higher than nuclear DNA (Brown *et al.*, 1979; Kunkel and Loeb, 1981; Miyata *et al.*, 1982; Niranjan *et al.*, 1982; Wallace *et al.*, 1987; Tritschler and Medori, 1993; Brown and Wallace, 1994; Torroni and Wallace, 1994; Kadenbach *et al.*, 1995; Yakes and Van Houten, 1997). This DNA damage is believed to be higher in the mitochondria than in the nucleus due to oxygen radicals generated as byproducts of OXPHOS. Unlike nuclear DNA, mtDNA lacks protective histone proteins and possesses an inefficient DNA repair system. Although early studies suggested that mitochondria possess no repair enzymes (Clayton *et al.*, 1974), it is now believed that mechanisms may exist in the mitochondria to conduct base-excision repair, but not nucleotide-excision repair or mismatch repair (Bogenhagen, 1999). Additionally, the mtDNA polymerase γ has a relatively high error insertion rate of 1/7000 bases (Kunkel and Loeb, 1981).

Due to the high copy number of mtDNA, a mutation in some of the mtDNA results in a mixture of variant mitochondrial genomes, a condition known as heteroplasmy (Holt *et al.*, 1990; Schon, 1993). Heteroplasmy is defined as the existence of two or more subpopulations of mtDNA genomes within a mitochondrion, cell, tissue, organ, or individual. In contrast, homoplasmy is defined as the condition where all mtDNA genomes have identical sequences. Heteroplasmy can be inherited through the female germ line, or can result following a somatic mutation during embryonic development or during an individual's lifetime. Once they arise, heteroplasmic populations must segregate during meiosis in the female germ line and during mitosis in the various somatic tissues.

Replication of mtDNA, as well as mitochondrial division, proceeds independently of nuclear replication and the cell cycle. During the ensuing cell division, mitochondria randomly segregate into daughter cells through a process known as replicative segregation (Wallace, 1992; Hamazaki *et al.*, 1993; Brown and Wallace, 1994; Johns, 1995). This process can result in a different proportion of mutant genomes being partitioned into each daughter cell. In somatic cells, the consequence may be a disease phenotype that varies over time (ie during development or the course of lifetime), or in space (ie among tissues) (Schon, 1993). In oocytes, the result may be marked variation in the heteroplasmic proportions among the offspring of a heteroplasmic woman. Studies at NIST have shown this wide variation of a heteroplasmy among 13 maternally related family members across three generations (Sekiguchi *et al.*, 1999).

The early studies of meiotic segregation of heteroplasmic variants focused on pedigree analysis of Holstein cows (Hauswirth and Laipis, 1982; Olivo *et al.*, 1983; Hauswirth *et al.*, 1984; Laipis *et al.*, 1988; Koehler *et al.*, 1991). In these studies, frequent transitions from homoplasmy in the mother to heteroplasmy in the offspring were observed, as well as occasional shifts from one apparent homoplasmic state to another in a single generation. Considering the large number of mtDNA genomes present in oocytes (> 100,000), these findings were puzzling and could not be explained by the same type of stochastic assortment of mtDNA genomes that is seen during somatic cell division. Therefore, a 'genetic bottleneck' was proposed to account for the rapid segregation of mtDNA genomes observed in these pedigrees (Hauswirth and Laipis, 1982; Koehler *et al.*, 1991). The bottleneck theory states that the number of mtDNA genomes is reduced to a relatively small number during some stage of oogenesis. Beyond this bottleneck, over-replication of the founder populations returns the total number of mtDNA molecules to its normally high level. Additional bottlenecks were thought to occur during embryogenesis, where only a small proportion of the mitochondria is incorporated into the developing embryo. Depending on the genomes that make up the founder population, such a bottleneck could result in a switch from one mtDNA genotype to another in a single generation, or cause an asymmetric distribution of mtDNAs within a set of offspring. In mitochondrial diseases, the bottleneck theory has been used to account for the marked variation in the proportions of mutant genomes commonly observed within a maternal lineage (Bolhius *et al.*, 1990; Poulton and Marchington, 1996; Marchington *et al.*, 1998).

In an attempt to gain a better understanding of the molecular basis of such a bottleneck, Jenuth *et al.* (1996) studied lines of heteroplasmic mice carrying two different mtDNA genotypes. Single cell PCR techniques were used to measure the proportions of both mtDNA genotypes at each stage of oogenesis as well as in somatic tissues in the offspring. The distribution of mtDNA genotypes in primary and mature oocytes from the same female did not significantly differ, nor did the distribution differ in the mature oocytes and F1 offspring derived from the same animal. These results suggested that the proportion of the mtDNA genotypes in the primary oocyte population was responsible for what was seen in offspring of the next generation, and that the bottleneck must therefore occur at an earlier stage in oogenesis. Analysis of primordial germ cells, which are present prior to the primary oocyte stage, demonstrated that the segregation pattern in these mice was due to random genetic drift which occurred very early in oogenesis, and not during maturation of oocytes or during early embryogenesis.

Mitochondrial DNA mutations in disease

The mtDNA coding region has been under extensive study since the late 1980s, when deletions and point mutations were found to be associated with neurodegenerative diseases (Holt *et al.*, 1988; Wallace *et al.*, 1988). Mitochondrial DNA diseases are rare, progressive, phenotypically heterogeneous disorders that primarily affect the more oxidative demanding organs, such as brain, eye, and skeletal muscle. However, although all pathogenic mtDNA mutations compromise some aspect of the OXPHOS process, they can result in a variety of disorders, such as epilepsy, blindness, strokes,

diabetes, and deafness. Affected individuals are commonly heteroplasmic for the mutation, with the phenotypic manifestation resulting from the type of the mutation (highly deleterious mutations result in earlier onset and more severe symptoms), the proportion and tissue distribution of the mutant genomes, and the age of the individual. One theory equates the progressive nature of mitochondrial diseases with the level of ATP production. Each tissue type requires a different minimum level (threshold) of ATP production for normal cellular function (Shoffner and Wallace, 1995; Wallace, 1995). When the proportion of mutant genomes causes ATP production to drop below this threshold, the disease phenotype will be displayed. Somatic mtDNA mutations, which accumulate during the ageing process, may further decrease the oxidative capacity of cells and thereby exacerbate an already existing illness or contribute to the ageing process. The nuclear genetic background of the individual has also been shown to influence the phenotypic display of the disease (Vilkki *et al.*, 1991). Additionally, a report of identical twins being discordant for the LHON phenotype, despite being genetically identical, suggests that epigenetic (eg environmental) factors may also play a role (Johns *et al.*, 1993).

Pathogenic point mutations in mtDNA have been found in the genes coding for proteins, as well as the tRNA and rRNA genes. Diseases resulting from point mutations are maternally inherited, and, with few exceptions, are found in the heteroplasmic state. Extreme variation in the severity of the phenotype has been found among siblings and maternal relatives (Bolhuis *et al.*, 1990; deVries *et al.*, 1994), presumably resulting in part from the bottleneck during oogenesis. Therefore, some investigators have correlated the severity or age of onset of the disease within a maternal lineage with the relative proportion of mutant genomes (deVries *et al.*, 1994; Olsson *et al.*, 1998). However, other studies have failed to establish this relationship (Tanno *et al.*, 1991; Piccolo *et al.*, 1993; Harrison *et al.*, 1997).

Leber's Hereditary Optic Neuropathy (LHON) was the first mitochondrial defect described which resulted from a point mutation (Wallace *et al.*, 1988). This disease is manifested by sudden onset of blindness at approximately 20 years of age. The majority of patients with LHON harbour a G → A transition at base pair position 11,778, which changes a conserved arginine of the NADH dehydrogenase sub-unit 4 (ND4) into a histidine. However, in other LHON patients, nucleotide substitutions have been identified in the ND1, ND2, ND5, ND6, cytochrome *c* oxidase, sub-unit 1 (CO1), and cytochrome *b* (Cyt *b*) genes (Lestienne and Bataille, 1994), making LHON the most genetically heterogeneous of the mitochondrial disorders. The Neuropathy, Ataxia, Retinitis Pigmentosa (NARP) syndrome results from a T → G transversion at base pair position 8,993, which changes a highly conserved leucine to arginine in the ATP synthase 6 gene or, less commonly, a T → C transition at this same position, which converts leucine to proline (Holt *et al.*, 1990; Schon *et al.*, 1994). Patients with NARP typically display a combination of developmental delay, retinitis pigmentosa, dementia, seizures, ataxia, proximal neurogenic muscle weakness, and sensory neuropathy. When the proportion of the T → G mutation is high (> 90%), it results in a lethal, multisystem degenerative disorder known as Leigh disease (Tatuch *et al.*, 1992; Takahashi *et al.*, 1998).

Mutations in the mitochondrial tRNA genes are likely to reduce protein synthesis either by incorrect folding or incorrect interaction with ribosomes. Patients with diseases resulting from tRNA mutations typically exhibit peripheral blotchy, red

patches called ragged red fibres in their skeletal muscle when tissue samples are stained with modified Gomori Trichrome stain. The ragged red fibres represent large accumulations of abnormal mitochondria, usually accompanied by decreased or absent cytochrome *c* oxidase activity (Shoubridge, 1994).

The disease called Myoclonic Epilepsy with Ragged Red Fibres (MERRF) has been shown to be associated with an A → G transition at position 8,344 and a T → C transition at position 8,356, both in the $tRNA^{Lys}$ gene (Schon *et al.*, 1994; Masucci *et al.*, 1995; Chomyn, 1998). Patients with MERRF typically demonstrate myoclonus, seizures, ataxia, and myopathy with ragged red fibres. Less common symptoms include dementia, short stature, hearing loss, neuropathy, and optic atrophy.

An A → G transition at 3,243 in the $tRNA^{Lys}$ gene is found in patients with the disease called Mitochondrial Encephalopathy, Lactic Acidosis, and Stroke-like Episodes (MELAS). Patients with MELAS typically experience strokes, encephalopathy characterized by seizures and dementia, lactic acidosis and ragged red fibres. Other symptoms sometimes include migraine headaches, vomiting, limb weakness, and short stature. (Tritschler and Medori, 1993; Brown and Wallace, 1994; Schon *et al.*, 1994). The 3,243 mutation accounts for the majority of MELAS cases; however, MELAS has also been associated with a T → C transition at position 3,271, and with an A → G transition at position 3,252, both also in the $tRNA^{Leu}$ gene. These mutations all appear in the heteroplasmic form, with high levels (> 80%) of mutant genomes in muscle (Schon *et al.*, 1994). Interestingly, an A → G transition at 3,243 has also been found to be associated with a form of maternally-inherited diabetes mellitus combined with sensorineural hearing loss (van den Ouweland *et al.*, 1992; Gerbitz *et al.*, 1995; Harrison *et al.*, 1997; Olsson *et al.*, 1998; van den Ouweland *et al.*, 1999). Patients displaying this phenotype typically lack the neurological symptoms seen with MELAS, despite having the same DNA mutation.

A heteroplasmic C → A transversion at position 12,258 in the $tRNA^{ser}$ gene was found in one family to be responsible for a phenotype similar to Usher Syndrome type III (Mansergh *et al.*, 1999), a disorder characterized by progressive hearing loss and retinitis pigmentosa starting at puberty. Usher Syndrome type III had been previously mapped to chromosome 3q and is normally transmitted in an autosomal recessive fashion. Members of this family additionally demonstrated marked variability in the severity of symptoms, a phenomenon that is commonly seen with mitochondrial disorders. Muscle biopsies from affected individuals also contained high levels of the 12,258 mutation, although clinically no muscle abnormalities were expressed.

Thus far, only one ribosomal RNA mutation has been associated with a mitochondrial disease. A point mutation (A → G) at position 1,555 in the 12S rRNA gene has been found to cause a rare form of maternally inherited deafness (Prezant *et al.*, 1993; Lestienne and Bataille, 1994; Fischel-Ghodsian *et al.*, 1997; Fischel-Ghodsian, 1998). This mutation appears in a highly conserved domain that is part of the aminoacyl site in which the mRNAs are decoded. This same mutation was also found to predispose individuals to aminoglycoside-induced hearing loss. Unlike other point mutations causing diseases, this mutation is found in the homoplasmic form (ie all cells harbour the mutation), and causes hypersensitivity to drugs such as streptomycin, kanamycin, and gentamycin (Schon *et al.*, 1994). In the United States, the 1,555 mutation accounts for approximately 15% of all cases of aminoglycoside-induced deafness (Fischel-Ghodsian, 1998).

Mitochondrial DNA rearrangements, including deletions, insertions, and duplications, may also cause disease. It is believed that the large number of direct repeats throughout the mtDNA genome contributes to the occurrence of deletions (Johns *et al.*, 1989). A slip-replication model, in which an upstream direct repeat of the parental heavy strand base pairs with the downstream light strand direct repeat during the replication process, has been proposed to cause a 4,977 base pair deletion (Shoffner *et al.*, 1989). Three types of disorders have been shown to result from mtDNA rearrangements: adult-onset diabetes and deafness, ocular myopathy, and a lethal form of childhood pancytopenia called Pearson's Marrow/Pancreas Syndrome. Diabetes and deafness has been associated with the presence of partially duplicated mtDNA, whereas large deletions (primarily the 4,977 base pair deletion) have been found to be the cause of the ocular myopathies and Pearson's Syndrome. The ocular myopathies include: (1) Chronic Progressive External Ophthalmoplegia (CPEO) (Hurko *et al.*, 1990), a relatively mild syndrome which includes ophthalmoplegia (paralysis of the eye muscles), ptosis (droopy eyelids), mitochondrial myopathy, and ragged red fibres; and (2) the more severe Kearns-Sayre Syndrome (KSS) which additionally features an early age of onset, atypical retinitis pigmentosa, mitochondrial myopathy, and usually one of the following: cerebellar syndrome, cardiac conduction abnormalities, or an elevated cerebral-spinal fluid protein (Shoffner *et al.*, 1989; Brown and Wallace, 1994). Pearson's Marrow/Pancreas Syndrome typically manifests as pancytopenia and pancreatic dysfunction due to the accumulation of deleted mtDNA genomes in the bone marrow precursor cells (Brown and Wallace, 1994; Wallace, 1994). Children surviving this disease often progress to KSS. Although patients with diabetes and deafness are normally members of maternal pedigrees exhibiting the same disease, CPEO, KSS, and Pearson's Marrow/Pancreas Syndrome usually occur as isolated cases, with the mother, siblings, and children of affected patients harbouring no detectable deleted mtDNA genomes (Larsson *et al.*, 1992; Brown and Wallace, 1994). However, these deletions *may* be maternally transmitted, since mitochondria in oocytes from healthy women have been found to contain measurable levels of such deletions (Chen *et al.*, 1995; Brenner *et al.*, 1998).

Mitochondrial DNA mutations have also been implicated in late-onset degenerative diseases such as Parkinson's disease and Alzheimer's disease (Brown and Wallace, 1994; Wallace, 1994; Wallace *et al.*, 1995). Three mutations may contribute to the etiology of these diseases. The first is a base substitution in the $tRNA^{Gln}$ gene at position 4,336 which converts a moderately conserved G to A. This mutation has been found at a statistically higher frequency in autopsy tissues from patients with these diseases than from normal controls (Wallace, 1994; Hutchin and Cortopassi, 1995). The second mutation is an ND1 base substitution at position 3,397, which converts a highly conserved methionine to valine. The third change consists of a five-nucleotide base pair insertion in the 12S rRNA gene, between nucleotides 956 and 965 (Wallace *et al.*, 1995).

The variation in the severity of the disease symptoms within a maternal lineage, as well as the inability to predict the patterns of tissue segregation, have been obstacles to providing effective prenatal genetic counselling to families with an identified pathogenic mtDNA mutation (Chinnery *et al.*, 1998; Poulton *et al.*, 1998). In most cases, it is not possible to predict the severity of the symptoms based on the proportion of mutant genomes in a chorionic villus sample. There is one report, however, in

which chorionic villus analysis from two separate pregnancies showed a high proportion of the 8,993 mutation associated with NARP in a woman who had previously delivered a severely affected child. Subsequent analysis of the foetal tissues from the abortions on both occasions demonstrated high levels of mutant genomes in several tissues, including muscle and brain, an indication that these offspring would also have been affected (Harding *et al.*, 1992; Poulton and Marchington, 1996). However, because of the ambiguities normally associated with the course of mitochondrial defects, prenatal genetic counselling of mitochondrial diseases is difficult to provide.

Replicative segregation of disease mutations

The majority of research involving replicative segregation of heteroplasmic variants in somatic tissues has been to better understand the mechanisms involved in the phenotypic expression of mitochondrial diseases. Several studies have shown that the mutation generally arises prior to the formation of the three germ layers (as evidenced by the different tissues where the mutation was observed) and subsequent replicative segregation of mutant and wild-type mtDNA genomes results in the variation of heteroplasmy noted among different tissue types. For example, Hamazaki *et al.* (1993) examined a series of organs from an individual with MELAS and found high levels of mutant mtDNA genomes in brain, skeletal muscle, heart, and liver, but lower levels of the mutation in the lung and spleen. In a study of patients with MELAS, deVries *et al.* (1994) found high levels of the mutation in skeletal muscle, but low levels in blood and fibroblasts. In a comparison of foetal and adult individuals affected with MELAS, Matthews *et al.* (1994) found very little variation among tissues from a 24-week old foetus, but wide variation in the proportion of the mutation among the adult tissues. Similar variation in heteroplasmic proportions have been reported in association with MERRF, where the highest levels of the mutation were found in the brain, heart, and skeletal muscle (Lertrit *et al.*, 1992) and LHON, where the highest levels were found in the skeletal muscle, heart, optic nerve, and retina (Howell *et al.*, 1994).

It is difficult to explain these patterns simply by random replicative segregation of heteroplasmic variants during development. Interestingly, a common finding is that the level of the mutation is highest in organs with the greatest oxygen demands. One hypothesis is that the relative abundance of mitochondria in the high demand oxidative organs or the proliferation of defective mitochondria in affected cells may contribute to the preferential increase of mutant mitochondria (Hamazaki *et al.*, 1993). Another theory proposes that rapidly dividing tissues such as leukocytes may have the ability to eliminate cells with the mutation, whereas postmitotic (ie non-dividing) tissues, such as brain and skeletal muscle, do not eliminate these cells and may instead favour the accumulation of mutant mtDNA genomes.

Additional evidence for nonrandom, tissue-specific drift of mtDNA genotypes in mice has been reported by Jenuth *et al.* (1997). These authors used strains of mice carrying non-deleterious coding region heteroplasmic variants to study segregation patterns among tissues. A series of tissues in newborn mice had similar proportions of the heteroplasmic species. However, significant differences were noted by one month of age in the liver, kidney, spleen, and blood. Specifically, the proportion of one

heteroplasmic species increased in the blood and spleen, whereas the proportion of the other species increased in the kidney and liver. This same pattern was found in strains of mice having different nuclear backgrounds, suggesting the existence of tissue-specific factors that may influence the segregation patterns of mitochondrial heteroplasmic polymorphisms.

Studies of transformed cell lines where mixtures of mutant and wild-type mtDNA genomes were introduced into ρ^o (mtDNA-depleted) cells have also suggested that segregation of mtDNA genotypes may not be stochastic in all cases. Yoneda *et al.* (1992) and Bentlage and Attardi (1996) studied the mutation leading to the disease MELAS and reported a unidirectional drift toward homoplasmy for the mutation after several cell divisions. Dunbar *et al.* (1995) expanded on these studies and found that the specific nuclear background of the cell line can influence the segregation pattern of the mutant and wild-type genomes. Although these authors observed an increase of the mutation leading to the disease MELAS with some nuclear backgrounds, they found random segregation of mutant and wild-type genomes with others. Holt *et al.* (1997) similarly found that the nuclear background influenced the segregation patterns of partially duplicated mtDNA genomes in cell culture. Shay and Ishii (1990) studied mtDNA segregation patterns by making somatic cell hybrids of HeLa cells with non-tumourigenic and tumourigenic cells. In the HeLa/tumourigenic hybrids, random mtDNA segregation occurred (ie stochastic segregation); whereas, in the HeLa/non-tumourigenic hybrids, the HeLa cell mtDNA was lost after several weeks of continuous cell culture (ie non-stochastic segregation).

Studies of human pedigrees in which disease mutations were segregating have suggested that the shifts in proportions of mtDNA genotypes between generations may not always be the result of random drift. For example, Smith *et al.* (1993) studied the mutation in base pair 11,778 associated with LHON and found that the percentage of mutant mtDNAs tended to increase in successive generations, indicating a unidirectional drift toward homoplasmy for the mutation. However, studies such as these are subject to ascertainment bias and therefore must be interpreted with caution. Ascertainment bias is the greater likelihood that those individuals displaying the phenotype would come to the attention of clinicians. Consequently, there would be little information regarding the families where the proportion of the mutant has *decreased* to subclinical levels in successive generations. Additionally, other studies of maternal pedigrees have demonstrated little to no shifts in the proportions of heteroplasmic variants among generations (Vilkki *et al.*, 1990; Howell *et al.*, 1992a; Lombes *et al.*, 1992; Piccolo *et al.*, 1993; Ghosh *et al.*, 1996).

Mitochondrial DNA mutations in ageing

In recent years, there has been a growing body of evidence suggesting a correlation between mtDNA mutations and normal ageing. The 'mitochondrial theory of ageing' states that deletions and point mutations accumulate in somatic cells during an individual's lifetime (Yen *et al.*, 1991; Cortopassi *et al.*, 1992; Sont and Vandenbroucke, 1993; Zhang *et al.*, 1993; Shigenaga *et al.*, 1994; Kadenbach *et al.*, 1995; Ozawa, 1995; Wallace, 1995; Wallace *et al.*, 1995; Jazin *et al.*, 1996; Weber *et al.*, 1997; Brierley *et al.*, 1998; Kovalenko *et al.*, 1998; Liu *et al.*, 1998; Papiha *et al.*, 1998; von Wurmb *et al.*, 1998; Cortopassi and Wong, 1999). These mutations are

believed to be due to damage from oxygen radicals generated by OXPHOS as well as other reactions taking place in the mitochondria. The resulting inhibition of the electron transport chain and reduction in ATP stimulates further free radical production, resulting in a vicious cycle. This process subsequently leads to impaired cellular respiration, which in turn leads to cell death and hence ageing.

Although several different deletions have been observed, a specific 4,977 base pair deletion (the 'common deletion' which is also seen in the diseases CPEO and KSS) has been frequently reported in postmitotic tissues (especially nerve and muscle) of healthy older people, but not in younger individuals or in foetal tissue (Cortopassi *et al.*, 1992; Cortopassi and Arnheim, 1993). A corresponding decrease in cellular respiratory function (as measured by assaying cytochrome *c* oxidase activity) in muscle fibres with high levels of deleted mtDNA in elderly individuals appears to corroborate this association (Brierley *et al.*, 1998). In contrast, the common deletion is found at much lower levels in rapidly dividing tissues (eg white blood cells) in normal elderly individuals (Yen *et al.*, 1991; von Wurmb *et al.*, 1998). It has been hypothesized that rapidly dividing tissues may eliminate respiratory deficient cells, whereas nondividing tissue may instead favour the accumulation of mutant mtDNAs.

In addition to deletions, point mutations have also been found to accumulate over time. Weber *et al.* (1997) reported an increase in the proportion of a mutation in the $tRNA^{Leu}$ gene over a 12-year period in skeletal muscle, but not blood, of a patient with progressive muscle weakness. A mutation at position 3,243, normally associated with MELAS, has also been found to accumulate in an age-associated manner (Zhang *et al.*, 1993; Lui *et al.*, 1997).

However, the association between mtDNA mutations and ageing is not without controversy, and some feel that there is little basis for a direct correlation between mtDNA lesions and cell senescence in aged subjects (Lightowlers *et al.*, 1999). Additionally, in contrast to other authors, Hayashi *et al.* (1994) reported that the decrease in oxidative function in cells from elderly individuals was due to an accumulation of nuclear recessive somatic mutations, as opposed to mitochondrial mutations.

Mitochondrial DNA in forensic analysis

In contrast with the conserved coding region, two areas of the noncoding or control region, termed hypervariable region 1 (HV1) and hypervariable region 2 (HV2), are highly polymorphic in humans (Aquadro and Greenberg, 1983; Greenberg *et al.*, 1983; Cann *et al.*, 1984; Horai and Hayasaka, 1990; Stoneking *et al.*, 1991). This high level of polymorphism, in conjunction with maternal inheritance and lack of recombination, make mtDNA analysis a useful tool for evolutionary studies and tracing the origins of various human populations (Cann *et al.*, 1987; Melton *et al.*, 1995; Melton *et al.*, 1997; Mumm *et al.*, 1997; Brown *et al.*, 1998; Comas *et al.*, 1998). Mitochondrial DNA analysis of the control region was used to develop the 'African Eve' hypothesis which states that all mtDNA types in contemporary populations can be traced back to a single African female ancestor who lived about 200,000 years ago (Vigilant *et al.*, 1991; Stoneking *et al.*, 1992; Stoneking, 1994).

The high mtDNA copy number, maternal mode of inheritance, and polymorphic control region additionally make mtDNA analysis a suitable tool for human

identification (Sullivan *et al.*, 1992; Wilson *et al.*, 1993; Holland *et al.*, 1995). The presence of multiple mtDNA genomes in each cell improves the likelihood of recovering sufficient DNA for analysis, particularly in cases where degradation or small DNA quantities prohibit the recovery of enough nuclear DNA for analysis. Since mtDNA is maternally inherited, an individual's mother, siblings, and all other maternally related family members are expected to have identical mtDNA sequences. These identifications can be verified by comparisons of mtDNA sequences from a questioned sample with sequences from any maternal reference sample, even if the unknown and reference individuals are separated by many generations. Forensic analysis typically involves extraction of total genomic DNA, followed by PCR amplification and sequencing of the HV1 (approximate nucleotide positions 16,024 to 16,365) and HV2 (nucleotide positions 73 to 340) regions (Sullivan *et al.*, 1991; Sullivan *et al.*, 1992; Ginther *et al.*, 1992; Holland *et al.*, 1993; Wilson *et al.*, 1993; Holland *et al.*, 1995). Nucleotide differences between the questioned sample and the Anderson reference sequence (see section on Genetic Properties of mtDNA) (Anderson *et al.*, 1981) are noted. A database consisting of mtDNA sequences from unrelated individuals is then used to predict the 'frequency' of the particular mtDNA genotype in the appropriate ethnic population. Sequence comparisons between the questioned sample and appropriate reference samples, collected either from the alleged contributor of the evidence or from maternal relatives, are also conducted.

Mitochondrial DNA analysis is becoming a common tool in many forensic laboratories, including the FBI and the Armed Forces DNA Identification Laboratory (Holland *et al.*, 1993; Holland *et al.*, 1995). Forensic mtDNA analysis has been successfully conducted on many tissue types in addition to the commonly used blood and skin. Some examples include: skeletal bones (Holland *et al.*, 1993; Gill *et al.*, 1994; Holland *et al.*, 1995; Ivanov *et al.*, 1996), teeth (Ginther *et al.*, 1992), hair (Wilson *et al.*, 1995; Allen *et al.*, 1998), fingernails (Kaneshige *et al.*, 1992; Roby *et al.*, 1996), and saliva (Allen *et al.*, 1998). Mitochondrial DNA analysis contributed to the determination of the geographic origin of the 5,000-year-old 'Tyrolean Ice Man' (Handt *et al.*, 1994), and was instrumental in the identification of the bones of Nicholas Romanov, the last Tsar of Russia, his wife and three daughters (Gill *et al.*, 1994; Ivanov *et al.*, 1996). The lack of a match also indicated that four sets of bones were from non-relatives of the Romanovs. The individual buried in the Tomb of the Unknown Soldier from the Vietnam War was also identified by mtDNA analysis (Daoudi, 1998; McElfresh and Holland, 1998). Since all military personnel are now required to supply a blood sample, which is stored in a repository, it is highly unlikely that there will be any more unknown U.S. soldiers in the future.

One disadvantage of using mtDNA for forensic purposes is the possibility that the occurrence of heteroplasmy will confuse the interpretation of the results and potentially lead to an erroneous exclusion of identity rather than a match. Early tissue studies of control region variants suggested that heteroplasmy in this area of the genome occurred rarely, if at all. Monnat and Reay (1986) isolated mtDNA from brain, heart, kidney, liver, and skeletal muscle from 2 individuals, then cloned and sequenced a 1,152 base pair fragment spanning nucleotide positions 41–1,193. This fragment contained a portion of the control region (including what is now referred to as HV2), as well as the phenylalanine tRNA gene and a portion of the 12S rRNA

gene. Base substitutions were observed between individuals and in comparison with the published Anderson reference sequence (Anderson *et al.*, 1981). However, no sequence differences were identified within or among tissues from the same individual (ie no heteroplasmy was observed). These results were in agreement with other studies that examined mtDNA isolated from blood (Monnat and Loeb, 1985) and retinal tissue (Bodenteich *et al.*, 1991). However, these studies were limited in scope, especially since: (1) very few individuals were examined; and (2) techniques were not available to detect low frequency mutations or polymorphisms present in low concentrations.

The first observation of heteroplasmy in the hypervariable region occurred in the analysis of the remains of Nicholas Romanov, the last Tsar of Russia. This case brought to light the impact that the presence of heteroplasmy could have on the resolution of a forensic case. In 1918, Nicholas, his wife Tsarina Alexandra, and their five children were executed by a Bolshevik firing squad. In 1991, nine skeletons were recovered from a shallow grave. These skeletons were tentatively identified by Russian forensic authorities as the remains of the Tsar, the Tsarina, three of their five children, the Royal Physician, and three servants. Subsequent mtDNA analysis revealed an exact sequence match between the putative Tsarina and the three children with a living maternal relative, Prince Phillip of England, whose grandmother was the Tsarina's sister (Gill *et al.*, 1994). However, the sequence analysis of the bones alleged to have originated from Nicholas revealed a C/T heteroplasmy (ie both cytosine and thymine were present) at position 16,169 in the HV1 region. Although the mtDNA sequences of two of his living maternal relatives matched the sequence of the bones at all other positions in HV1 and HV2, they did not have the heteroplasmy at 16,169. Both reference individuals were homoplasmic for thymine at 16,169. This discrepancy caused some to question the authenticity of the identification. To rectify this, the remains of Nicholas's younger brother, Georgij, were exhumed. Mitochondrial DNA sequencing revealed that Georgij was also heteroplasmic at position 16,169, thereby confirming the identification of the Tsar (Ivanov *et al.*, 1996). This example demonstrates that, while the presence of heteroplasmy can increase the power of the match when present in both the questioned and reference samples, it can also confuse interpretation when it exists in one sample, but not the other.

In more recent years, researchers have detected heteroplasmy in the noncoding region of the mtDNA of different tissues from apparently normal individuals; these tissues include blood (Bendall *et al.*, 1996; Parsons *et al.*, 1997), hair (Comas *et al.*, 1995; Sullivan *et al.*, 1996; Bendall *et al.*, 1997; Wilson *et al.*, 1997), and brain (Jazin *et al.*, 1996). Additionally, vast differences in the proportions of heteroplasmic variants have been found among hairs from the same individual (Bendall *et al.*, 1997; Wilson *et al.*, 1997), as well as an apparent homoplasmic sequence difference between hair and a reference saliva sample collected from the same individual (Sullivan *et al.*, 1996). A recent study used a sensitive denaturant gradient gel electrophoresis (DGGE) assay and found low levels of HV1 heteroplasmy in post-mortem tissues, including bone, brain, liver, muscle, hair, and blood in 11 out of 21 normal adults (Tully, 1998). To address the heteroplasmy issue, the forensic community has adopted a policy of reporting a result as 'inconclusive' when only a single base difference is noted between the questioned and reference samples. Also, when possible, additional reference specimens, such as hairs or buccal swabs, are collected

and examined in order to obtain more comprehensive information and possibly reach a conclusive result.

Hypervariable region 1 length heteroplasmy

Hypervariable region 1 contains a homopolymeric tract of cytosines (C) between positions 16,184 and 16,193, which is normally interrupted at position 16,189 by a thymine (T). However, a T → C transition at 16,189, which is found in approximately 12–15% of the population (Bendall and Sykes, 1995), results in an uninterrupted 'C-stretch'. Although the predicted length of the C-stretch is 10 nucleotides (16,184 through 16,193), most individuals with the 16,189 transition have homopolymeric tracts of several different lengths (ie length heteroplasmy) (Horai and Hayasaka, 1990; Bendall and Sykes, 1995; Levin *et al.*, 1994, 1996; Marchington *et al.*, 1996). Cloning experiments following PCR have revealed lengths ranging from eight to 14 nucleotides (Bendall and Sykes, 1995), although tracts are predominantly 10, 11, or 12 nucleotides long. This length heteroplasmy is believed to be caused by replication slippage, which has been observed at poly G:C tracts (Hauswirth *et al.*, 1984). Direct sequencing of DNA from these individuals demonstrates reading frame shifts and subsequent inability to accurately sequence past the C-stretch. Bendall and Sykes (1995) examined the C-stretch heteroplasmy using PCR to amplify their DNA samples and their clones, whereas Levin *et al.* (1996) showed the C-stretch length heteroplasmy by cloning the DNA directly from tissue culture cells containing the C-stretch. Levin *et al.* (1996) also found that PCR of the cloned product introduced additional errors. Different clones had different numbers of C residues but, regardless of the number of C's, the sequence in the clones following the C-stretch could be determined without any problem (ie there was no replication slippage). To obtain sequence information from both strands immediately following the C-stretch without cloning, additional sequencing reactions can be performed with primers that sit on the C-stretch.

Molecular analysis of mitochondrial DNA mutations

Several molecular methodologies have been successfully applied to detect mtDNA sequence variation (*Table 6.3*) (Butler and Levin, 1998). The advent of PCR has greatly facilitated mtDNA analysis, since it is no longer necessary to perform the tedious task of separating cellular nuclear and mitochondrial DNA fractions prior to analysis. Although sequencing an entire region of DNA provides the most comprehensive genetic information, a number of screening assays have been successfully employed for detecting single base pair changes [eg single nucleotide polymorphisms (SNPs) and deleterious point mutations], deletions, and insertions. For example, denaturant gradient gel electrophoresis (DGGE) (Lerman and Silverstein, 1987; Cariello and Skopek, 1993) and single strand conformational polymorphism (SSCP) analysis (Orita *et al.*, 1989) can be optimized to detect the presence of a mutation anywhere within a specified DNA fragment. Although these methods provide little information regarding the exact position of the nucleotide change that occurred, they serve as valuable screening assays and allow high throughput analysis. Once a nucleotide change is detected, that sample can be further analysed by sequencing to

Table 6.3. Methods used for analysis of mtDNA variation

Assay	Principle	Region Analysed	Detection of Disease Mutation
Sequence-specific oligonucleotide probes (SSO)	PCR amplification followed by hybridization and detection of single base changes using oligonucleotide probes.	Control region (Melton *et al.*, 1997).	MELAS, LHON, NARP (Wong and Senadheera, 1997). LHON (Schollen *et al.*, 1997).
PCR using a mismatched primer	Mismatched primer creates restriction site in mutant genomes.		MERRF (Tanno *et al.*, 1991).
Minisequencing	Incorporation of single normal or mutant nucleotide identifies specific mutation.		LHON (Juvonen *et al.*, 1994).
Allele-specific PCR	Mutation-specific base in 3' position in primer allows only mutant genomes to be amplified.		MELAS (Zhang *et al.*, 1993; Liu *et al.*, 1997). LHON (Norby, 1993).
Denaturant gradient gel electrophoresis (DGGE)	Sequence-dependent differences in melting temperature (Tm) allow separation of mutant and wild-type sequences in a chemical denaturant gradient.	Control region (Hanekamp *et al.*, 1996; Jazin *et al.*, 1996; Steighner *et al.*, 1999) 22 tRNA genes (Michikawa *et al.*, 1997) ND1, COI (Hanekamp *et al.*, 1996).	MELAS (Lombes *et al.*, 1992).
Constant denaturant capillary electrophoresis (CDCE)	Capillary-based modification of DGGE. Mutant and wild-type sequences are separated from each other in an acrylamide-filled capillary using constant denaturant conditions.	206 base pair coding region fragment (Khrapko *et al.*, 1994).	
Single-strand conformation polymorphism (SSCP)	Different conformations of mutant and wild-type single-stranded products result in different electrophoretic mobility in a non-denaturing gel.	Control region (Kim *et al.*, 1995; Alonso *et al.*, 1996; Alonso *et al.*, 1997) $tRNA^{Ile}$, cyt *b* (Kim *et al.*, 1995).	MERRF (Sherratt *et al.*, 1996). LHON (Jaksch *et al.*, 1995; Mashima *et al.*, 1995). MELAS (Thomas *et al.*, 1994; Jaksch *et al.*, 1995).
Quantitative PCR	Simultaneous use of three primers amplifies mutant and wild-type genomes in a quantitative manner.	4977 bp deletion (Sciacco *et al.*, 1994).	
DNA array	135,000 probes complementary to entire mtDNA genome contained on a chip.	Entire mitochondrial genome (Chee *et al.*, 1996).	
Low-stringency single specific primer PCR (LSSP-PCR)	DNA target region is amplified by PCR. Second round PCR using single primer and low stringency generates profile.	Control region (Pena *et al.*, 1994; Barreto *et al.*, 1996; Marino *et al.*, 1996).	

identify the exact nucleotide and its location. Other assays use sequence-specific oligonucleotide (SSO) probes to detect single base changes. SSO probes can detect specific mutations without the need for sequencing, although probes must be designed to detect each individual polymorphism. However, this technology is amenable to multiplexing and automation; for example, Chee *et al.* (1996) published results on the development of a DNA array (chip) containing 135,000 probes to examine the entire mtDNA genome.

The presence of heteroplasmy is an additional factor that must be considered when selecting the appropriate method for analysing mtDNA variation. With direct sequencing, it is difficult to discern heteroplasmy from background artifacts. Additionally, automated sequencing chemistries that use fluorescently tagged terminators incorporate individual terminators into the sequencing reaction with disproportionate efficiencies. This is due in part upon the sequence of the DNA template itself (Parker *et al.*, 1995; Parker *et al.*, 1996), but results in further confounding the interpretation of heteroplasmic mixtures. Cloning PCR products or DNA directly is not subject to the same limitations as direct sequencing; however, cloning, followed by sequencing all of the clones to detect low levels of heteroplasmy is very labour intensive.

Several quantitative methods have been developed to detect a heteroplasmy present at low concentrations. Many have shown superior sensitivity over sequencing, which typically requires that the minor heteroplasmic sequence be present at a minimum concentration of 10–20% (Bendall *et al.*, 1996). For example, a DGGE assay designed to examine heteroplasmy in hypervariable region 1 (HV1) demonstrated sufficient sensitivity to detect a heteroplasmic species present at concentrations as low as 1% (Tully, 1998; Steighner *et al.*, 1999). Detection and quantification of the 11,778 mutation (LHON) at levels as low as 1.5% has been achieved using DNA minisequencing (Juvonen *et al.*, 1994). Constant denaturant capillary electrophoresis (CDCE), a capillary-based modification of DGGE, has been shown to detect heteroplasmic sequences present at less than 0.03% (Khrapko *et al.*, 1994).

Standard reference materials

The rapid evolution of molecular biology methodologies accentuates the need for quality assurance in the areas of both diagnostic testing and forensic identity determinations. Appropriate controls and standards are necessary to ensure that the data being collected, published, and entered into massive databanks and on the Internet are accurate. One of the avenues for providing the scientific community with the quality assurance that the sequence information is correct is using Standard Reference Materials (SRMs) as controls or calibration tools. The National Institute of Standards and Technology (NIST) has developed three Standard Reference Materials (SRM 2390, SRM 2391, and SRM 2392) to provide quality assurance in the analysis of human DNA. Two of these SRMs – the DNA Profiling SRM 2390 and the polymerase chain reaction (PCR)- based DNA Profiling SRM 2391 – provide quality control for the screening assay called Restriction Fragment Length Polymorphism (RFLP) and for the use of PCR-based genetic testing, respectively. Both use nuclear DNA and are intended for use in forensic and paternity identifications, for instructional law enforcement, or non-clinical research purposes. The third NIST SRM – 2392 – is to provide standardization and quality control for those doing PCR and

sequencing of any segment or the entire human mtDNA (16,569 base pairs) (Levin *et al.*, 1999). In addition, NIST is currently working on the development of a heteroplasmic mtDNA SRM which will provide quality control and assurance to those scientists who are trying to examine and detect mtDNA mutations present in low concentrations (Tully *et al.*, 1999).

Conclusions

This chapter has reviewed some of the current areas of scientific interest with regard to human mitochondrial genetics. For more information, the authors recommend the recent issue of the journal *Science*, volume 283, March 5, 1999, in which many articles are devoted to current research on mitochondria. In addition, a number of World Wide Web sites provide a vast array of information. Some of the main sites include: http://infinity.gen.emory.edu/mitomap.html; http://www.ncbi.nlm.nih.gov/htbin-post/Entrez/query?uid=1944628&form=6&db=n&Dopt=g; http://shelob.bioanth.cam.ac.uk/mtDNA/; http://www-lecb.ncifcrf.gov/~zullo/migDB/.

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