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Genetic Selection in Human Mitochondria

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ACADEMIC DISSERTATION

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To the people who raised me:
Tuula Maksimo
and my parents

Once upon a time in a distant corner of the known universe there was a planet. Life on this planet arose from the Egg of the Holy Chicken. Mitochondria were descendants of the feathers of the Holy Chicken.

Contents

List of original publications

This dissertation is based on following original communications. They are referred to their Roman numerals I-V in the text. In addition, some unpublished results are shown.

I El Meziane A*, **Lehtinen SK***, Hance N, Nijtmans LG, Dunbar D, Holt IJ & Jacobs HT (1998). A tRNA suppressor mutation in human mitocondria. *Nature Genetics* 18: 350-353. (* equal contribution)

II El Meziane A, **Lehtinen SK**, Holt IJ & Jacobs HT (1998). Mitochondrial tRNA Leu isoforms in lung carcinoma cybrid cells containing the np 3243 mtDNA mutation. *Human Molecular Genetics* 7: 2141-2147.

III **Lehtinen SK**, Hance N, El Meziane A, Juhola MK, Juhola KMI, Karhu R, Spelbrink JN Holt IJ & Jacobs HT (2000). Genotypic stability, segregation and selection in heteroplasmic human cell lines containing np 3243 mutant mtDNA. *Genetics* 154: 353-380.

IV Jacobs HT, **Lehtinen SK** &Spelbrink JN (2000). No sex please, we're mitochondria: a hypothesis on the somatic unit of inheritance of mammalian mtDNA. *BioEssays* 22: 564-572.

V **Lehtinen SK**, Spelbrink JN & Jacobs HT (2001). Heteroplasmic segregation associated with trisomy-9 in cultured human cells. Submitted for publication in *Molecular Biology of the Cell*.

Abbreviations

ADP	adenosine diphosphate
ATP	adenosine triphosphate
CGH	comparative genomic hybridization
Cot-1 DNA	DNA reannealed to C_0t=1.0, i.e. consisting of highly repetitive sequences
DAPI	4,6-diamidine-2-phenylindole hydrochloride
D-loop	displacement loop
DMEM	Dulbecco's modified Eagle's medium
EF-Tu	translational elongation factor Tu
FAD	flavin-adenine dinucleotide
FCS	fetal calf serum
FISH	fluoresence *in situ* hybridization
FITC	fluorescein isothiocyanate
GDP	guanosine diphosphate
GTP	guanosine triphosphate
HSP	heavy strand promoter
H-strand	heavy strand
IM	inner membrane
IMS	inter-membrane space
KSS	Kearns-Sayre syndrome
LHON	Leber's hereditary optic neuroretinopathy
LSP	light strand promoter
L-strand	light strand
MELAS	mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes
MERRF	myoclonic epilepsy and ragged-red fibres
mRF-1	mitochondrial release factor 1
mRNA	messenger RNA
mtDNA	mitochondrial DNA
mTERF	mitochondrial transcriptional termination factor
mtTFA	mitochondrial transcription factor A
NAD	nicotinamide adenine dinucleotide
NARP	Neuropathy, ataxia and retinitis pigmentosa
ND	NADH dehydrogenase
np	nucleotide position
OM	outer membrane
OXPHOS	oxidative phosphorylation
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PBS	phosphate-buffered saline
PEO	progressive external ophthalmoplegia
Pi	inorganic phosphate
PMSF	phenyl-methyl-sulfonyl-fluoride

POLG	DNA polymerase γ
rRNA	ribosomal RNA
RT	room temperature
SDH	succinate dehydrogenase
SDS	sodium dodecylsulphate
tRNA	transfer RNA
2-D	2-dimensional

ABSTRACT

Mitochondrial mutations are involved in a number of diseases in humans. One of the commonest classes of these mutations is in the gene for tRNA-Leu(UUR). I have studied the A3243G mutation in human cybrid cells. This mutation can cause a wide variety of disorders, varying from diabetes and/or deafness, skeletal or ocular myopathy to the full MELAS syndrome (mitochondrial myopathy, encephalopathy, lactic acidosis and strokelike episodes). In lung carcinoma cybrid cells, the A3243G mutation was found to be associated with a decreased level of both the amount of tRNA-Leu(UUR) and the extent of its aminoacylation. This was accompanied by both quantitative and qualitative defects in mitochondrial protein synthesis and poor respiration of the cells. I identified a cybrid cell-line having 99% mutant mtDNA at np 3243 but with a normal cellular phenotype. This phenotypic suppression was shown to be associated with heteroplasmy for another point mutation in mtDNA (G12300A), in the anticodon of tRNA-Leu(CUN). The G12300A mutation creates a suppressor tRNA able to read Leu(UUR) codons. This is the first suppressor mutation of its kind to be described in mammalian cells. It provides strong evidence that the protein synthesis defect caused by the A3243G mutation is due primarily to a deficiency of Leu(UUR) decoding capacity, at least in the lung carcinoma background. In addition, I have studied mtDNA segregation in heteroplasmic cells containing the A3243G mutation, with or without the G12300A suppressor. The number of independently segregating units was found generally to be greater than 1000, regardless of the presence or absence of the G12300A suppressor, i.e. at least an order of magnitude greater than described earlier in the mouse germ-line. The number of mtDNA nucleoids in the same cells was estimated to be approximately 50-100, based on the use of a transiently expressed reporter protein targeted to nucleoids. These studies have given rise to a new model for mtDNA organization and maintenance in the cell, based on the idea of faithfully duplicated mtDNA nucleoids that can themselves be heteroplasmic. One sub-culture of the cell-line containing the G12300A suppressor mutation was found to have undergone a transient episode of much faster mitotic segregation, resulting in diverse heteroplasmy levels. This mitochondrial genomic instability was shown to be associated with the gain of an additional copy of chromosome 9. Cells with trisomy 9, that had experienced transient mitotic segregation but had now re-established stable heteroplasmy, had the same number and subcellular distribution of mitochondrial nucleoids as cells containing two copies of chromosome 9, and which had exhibited heteroplasmic stability continuously. These findings reveal important effects of both the nuclear and mitochondrial genomes in modifying the phenotypic effects of a mtDNA disease mutation.

1. Introduction

Abnormalities in mitochondrial function are related to several different kinds of diseases in humans (Grossman and Shoubridge 1996, Suomalainen 1997). These abnormalities can be caused by mutations in either mitochondrial or nuclear genes or in a combination of both. A given disease can be inherited by normal Mendelian genetics (nuclear mutations) or as a non-mendelian trait showing maternal inheritance (mitochondrial mutations), or the disease can be sporadic. The transmission of mitochondrial disease has been widely studied, but surprisingly little is known about the transmission of mitochondrial DNA, especially in somatic cells, or about the subcellular organization of the mitochondrial genome and its distribution in the tissues of an individual. New genes involved in mitochondrial function are found frequently, as are new disease-causing mutations in mitochondrial DNA (mtDNA).

An important class of mutations in mtDNA causing disease are the tRNA mutations. One particular tRNA, tRNA-Leu(UUR) seems especially frequently to accumulate mutations that cause disorders in humans. These mutations cause a wide variety of diseases, varying from diabetes and/or deafness, skeletal or ocular myopathy or cardiomyopathy to the full MELAS syndrome (mitochondrial myopathy, encephalopathy, lactic acidosis and strokelike episodes). It has been puzzling for a long time, how a single mutation, for example, the A3243G mutation in tRNA-Leu(UUR), can cause such a wide variety of different phenotypes. One possible explanation is the difference in nuclear background interacting with mitochondria. Since most of the genes needed for mitochondrial maintenance and function are encoded by nuclear DNA, it is very likely that differences in nuclear genes have an effect on the phenotypic expression of mtDNA mutations. The molecular mechanism by which mitochondrial tRNA mutations such as A3243G produce cellular dysfunction also remains controversial.

The other issue of which we have surprisingly little knowledge and understanding is the maintenance and segregation of the mitochondrial genome. Mitochondrial DNA accumulates mutations at a much higher rate than nuclear DNA, and is thus highly polymorphic in the human population. Even though mtDNA is present in a single cell as a multicopy molecule, in the range 10^3-10^4 copies per cell, it is normally homoplasmic, i.e. all the copies of mtDNA are identical. However, in the presence of many mitochondrial mutations causing disease, the state of the mitochondrial genome is heteroplasmic, i.e. both mutated and wild type DNA molecules coexist in a single cell. We know that the amount of mtDNA is normally kept constant from one cell generation to the next, except where cells are undergoing differentiation. In heteroplasmic cells, it is possible that selection

works to reduce the amount of mutated DNA, as is generally accepted to be the case in blood cells. However, in post-mitotic cells, since there is no cell division, selection can only operate, if at all, at the level of the organelle. In contrast to negative selection in blood, disease mutations can actually accumulate with age in many cases. An exception occurs when the satellite cells in skeletal muscle do not contain mutated DNA, even though it is present in the differentiated muscle cells. It is therefore of the utmost important to understand how the mitochondrial genome segregates between cell generations and tissues of an individual.

In this thesis I will briefly describe the common features behind mitochondrial biogenesis and disease. I will focus on human mitochondrial biology mentioning only a few examples from other organisms. The thesis concentrates on three issues: the cellular defects arising from a mitochondrial tRNA disease mutation, the suppression of this pathological phenotype in a cell culture model and the segregation of mutated DNA molecules in cell culture. The experimental work that I will describe gives some very strong indications of the kinds of genetic factors that influence mitochondrial disease phenotype. However, it also reveals some limitations of the cell culture models that I have used, and which are also used by most other investigators in the field.

2. Review of literature

2.1 General aspects of mitochondria

All eukaryotic cells that respire contain mitochondria. Mitochondria have for long been known as "the powerhouses of the cell", thus providing ATP, the form in which energy is made available for the diverse uses of cells. Only during the last few decades have people realized the meaning of mitochondria in other cellular functions as well, for example, apoptosis (see review Desagher and Martinou 2000), cell division (von Wangenheim and Peterson 1998), and possibly aging (Melov 2000, Rustin et al 2000). The role of mitochondria in different kinds of human diseases has also begun to be understood.

Mitochondria are now generally believed to have originated by a symbiotic relationship between two types of bacteria: a protoeukaryote without mitochondria and a proteobacterium with respiration but a much simpler internal cellular organization. This process is called endosymbiosis, because one partner (the former) is proposed to have engulfed the other, which eventually became mitochondria. The full sequence of events proposed by this theory has been extensively discussed by Margulis (1981) and further described later (Brown and Doolittle 1997, Gray et al 1999, Lang et al 1999).

Mitochondria provide energy for the use of the cells via oxidative phosphorylation. They contain the proteins needed for cellular respiration, the enzymes of the citric acid cycle and in addition the enzymes needed for fatty acid oxidation. The proton motive force produced by the electron transport chain is used by ATP synthase to make ATP from ADP and inorganic phosphate. ATP is the cell's major form of usable energy, by virtue of the high energy (third) phosphodiester bond whose hydrolysis can energize many biochemical reactions (see Nicholls and Ferguson 1992). The other important metabolic functions of mitochondria are the breakdown of fatty acids via β-oxidation and the breakdown of some amino acids. Mitochondria are also involved in other important cellular functions, for example Ca^{2+}-homeostasis, synthesis of Fe/S clusters and apoptosis.

2.2 Structure and dynamics of mitochondria

2.2.1 The structure of mammalian mitochondria

Mitochondria have two membranes, the outer (OM) and inner (IM) membranes. In the old-fashioned view, based mainly on electron microscopy studies, the inner membrane is folded to create cristae. The inter-membrane space (IMS) is located between the membranes, and the matrix is enclosed by the inner membrane. The shape and number of mitochondria are different between cell types and according to different energetic demands upon the cell. Nowadays, with the emerging view of mitochondria as a dynamic network, it is better to refer to the mitochondrial volume (or mitochondrial membrane area) of the cell.

Based on electron microscope tomography, the IM can be divided into two distinct domains: an inner boundary membrane and cristal membranes (Perkins et al 1997, Frey and Manella 2000, Perkins and Frey 2000). The inner boundary membrane is located close to the OM and makes close contacts with it at numerous positions. On the other hand, the cristae form lamellar structures that are connected to the inner boundary membrane by small tubular structures called crista junctions (Perkins et al 1997).

Mitochondria are attached to the cytoskeleton. In yeast cells the actin cytoskeleton plays an important role (see review by Yaffe 1999). Several proteins involved in mitochondria-actin filament binding have been identified so far. Mitochondrial outer membrane proteins Mmm1p and Mdm10p are an essential link between mitochondria and the cytoskeleton (Boldogh et al 1998). Mitochondrial fusion is mediated by a GTP-binding protein Fzo1p (Hales and Fuller 1997, Hermann et al 1998) and fission reguires at least a dynamin-related protein Dnm1p (Otsuga et al 1998) and Gag3p (Fekkes et al 2000). Yeast dynamin-like proteins, i.e. Mgm1p are involved in yeast mitochondrial inheritance (Shepard and Yaffe 1999). Much less is known about mitochondrial attachment to the cytoskeleton in mammalian cells but, in contrast to yeast, the microtubular network plays an essential role in controlling the distribution of mitochondria (Heggeness et al 1978). In mammalian cells members of the kinesin protein family, Kif1B (Nangaku et al 1994) and Kif5B (Tanaka et al 1998) are responsible for the movement of mitochondria along the microtubules. Induced depolymerization of microtubules leads to inhibition of mitochondrial volume and mass increases during interphase (Karbowski et al 2001). On the other hand, stabilization of microtubules causes the accumulation of mitochondria (Karbowski et al 2001). However, the exact protein composition mediating the attachment between mitochondria and microtubules, and the role of these proteins, if any, in organizing the mitochondrial DNA, remains to be elucidated.

2.2.2 Mitochondrial network

Mitochondria are not static "tubes" inside the cell. Instead they form a highly dynamic network ("mitochondrial reticulum") with continuous fission and fusion (Bereiter-Hahn and Voth 1994, Nunnari et al 1997).

However, this continuous fusion seems not to be involved in mixing mtDNA between organelles. It has been shown that yeast mitochondrial proteins can move freely from one mitochondrion to another, but that is not the case with DNA (Newman et al 1996, Nunnari et al 1997). Also there is evidence that human mitochondria behave similarly. Moraes et al (1999) showed that in human-ape cell fusions the human nucleus imposes a strong preference for the mtDNA of its own species. This can be explained on the basis that the nuclear-coded mitochondrial replication factors may have a strong preference for human mtDNA, and that these factors are able to move from one mitochondrion to another. In contrast, it has been inferred that mtDNA cannot move freely between organelles since mitochondria carrying different mutations cannot easily complement one another (Attardi et al 1995, Enriquez et al 2000). In addition, there is no convincing evidence of intermolecular recombination, which would require mtDNA movement (Tang et al 2000a).

2.2.3 Proteins of the respiratory chain and ATP synthase

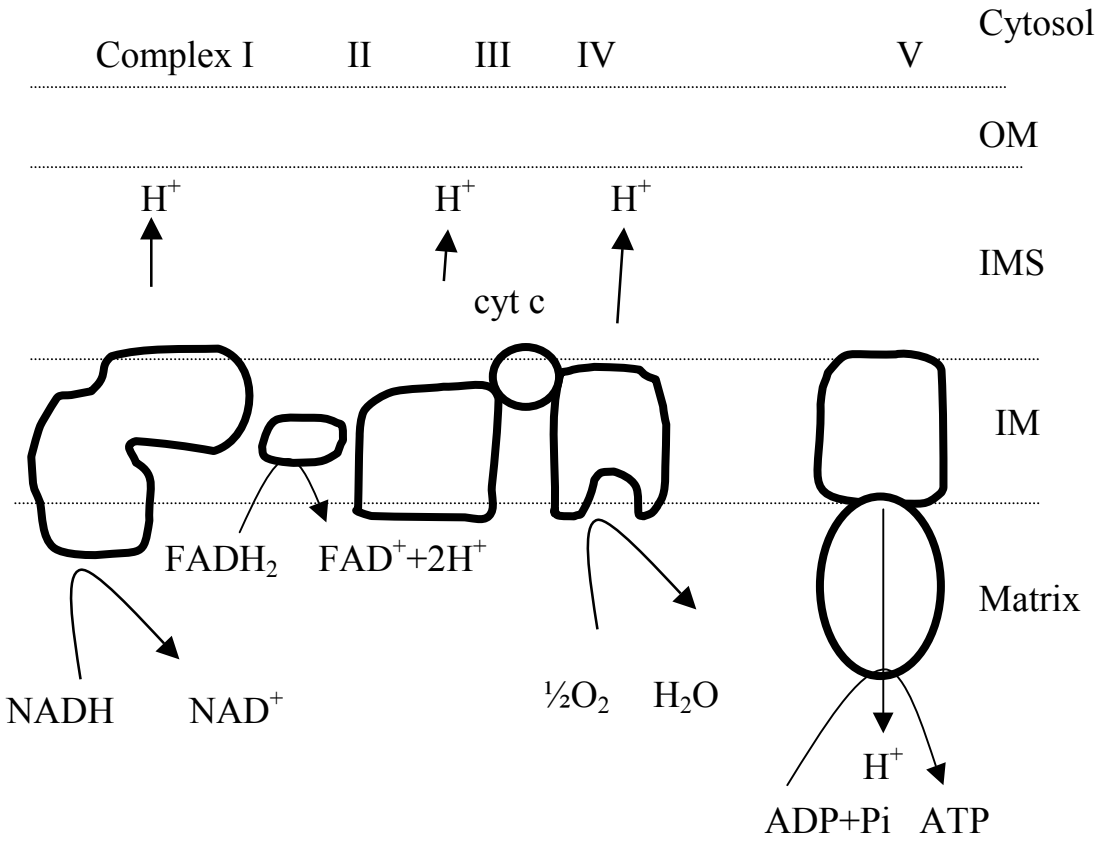


Figure 2.1. Proteins involved in oxidative phosphorylation and ATP synthesis. Modified from Nicholls and Ferguson (1992).

The proteins involved in cell respiration and ATP synthesis are located in the inner membrane. The following protein complexes are involved in cell respiration and ATP synthesis:

Complex I (NADH-ubiquinone oxidoreductase) (EC 1.6.5.3) has at least 42 subunits, of which seven are encoded by mtDNA (Pilkington 1993, Smeitink et al 1998). It transfers protons to the IMS, linked to the oxidation of NADH by ubiquinone.

Complex II (succinate dehydrogenase) (EC 1.3.99.11) is the smallest and simplest of all of the complexes. It is not involved in proton pumping, instead it transfers electrons from succinate to ubiquinone. The subunits of complex II are all encoded by the nucleus in humans (Hederstedt and Rutberg 1981, Scheffler 1998)

Complex III (cytochrome *bc*1 complex) (EC 1.10.2.2) has eleven subunits in humans, of which only one, cytochrome *b* is encoded by mtDNA. The other important subunits are the Rieske protein (an iron-sulphur protein) and cytochrome *c*1 (Trumpower 1990). Complex III catalyses the reduction of cytochrome *c* by ubiquinol in association with proton translocation. Cytochrome *c* is located between complexes III and IV on the outer face of the IM, and it transfers electrons from complex III to complex IV.

Complex IV (cytochrome *c* oxidase) (EC 1.9.3.1) is the terminal electron acceptor. It reduces molecular oxygen to water and translocates protons across the membrane. Three of its 13 protein subunits are encoded by mtDNA. The bovine complex IV structure has been solved (Tsukihara et al 1995, 1996).

Complex V (ATP synthase) (EC 3.6.1.3) catalyses the synthesis of ATP from ADP and inorganic phosphate. This reaction is driven by translocation of protons down the proton gradient created by complexes I, III and IV. The enzyme consists of two parts: the membrane segment, F_O, and the F_1 part that faces towards the matrix (Abrahams et al 1994). The F_1 subcomplex comprises subunits designated α, β, γ, δ and ε, assembled with the stoichiometry 3:3:1:1:1. Subunits δ, γ and ε form the central stalk of ATP synthase that is a key rotatory element of the enzyme (Collinson et al 1996, Gibbons et al 2000). The structure of the F_O domain of the enzyme is less well-defined, but it is known to comprise subunits a (or A6) and A6L (or A8), both of which are mtDNA-encoded, plus nuclear-coded subunits b, c and d, plus others. 6-7 subunits c are in close contact with the central stalk. The rotation of F_O is driven by the proton motive force which energises the release of ATP from its binding sites in the F_1 portion of the enzyme, via conformational changes (Stock et al 1999).

2.3 The mitochondrial genome

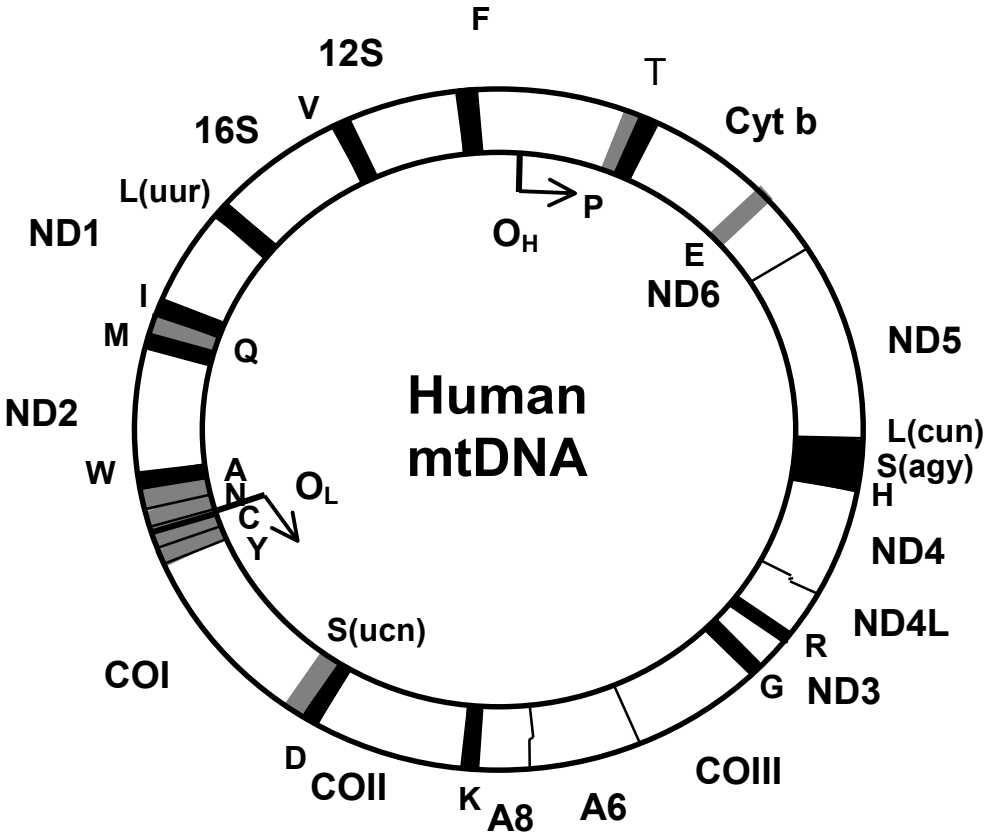


Figure 2.2. Human mitochondrial genome. Genes encoded by human mtDNA are ND, NADH dehydrogenase; CO, cytochrome oxidase; A, ATP synthase; cyt b, cytochrome b. In addition, two ribosomal RNAs, 12S and 16S are encoded by human mtDNA. tRNAs are scattered throughout genome. tRNAs are F, phenylalanine; V, valine; L, leucine; I, isoleucine; M, methionine; W, Tryptophan; A, alanine; N, asparagine; C, cysteine; Y, tyrosine; S, serine; D, aspartic acid; K, lysine; G, glycine; R, arginine; H, histidine; E, glutamic acid; T tyrosine and P, proline. The light-strand encoded tRNAs are shown in grey. Also ND6 is encoded by the light-strand. O_H is the origin of heavy strand replication, O_L is the origin of light strand replication.

The human mitochondrial DNA is a closed circular molecule of size 16.6 kb and it was the first human chromosome to be completely sequenced, in 1981 (Anderson et al 1981). It is very compact, containing little non-coding sequence, essentially just the 1 kb D-loop region, and having even some overlapping genes. The non-coding region that includes the D-loop is located between two tRNA genes, F and T. O_H is the origin of heavy strand replication, O_L is the origin of light strand replication.

2.3.1 Genes for structural proteins

mtDNA codes for 13 subunits of the respiratory chain. Subunits I, II and III of cytochrome *c* oxidase are encoded by *COXI*, *COXII* and *COXIII*. One subunit of cytochrome *b* is encoded by the *CYTB* gene. Seven subunits of NADH dehydrogenase are encoded by *ND1-6* and *ND4L* (Attardi et al 1986, Chomyn et al 1986). The F_O portion of the ATP synthase has two mitochondrially encoded subunits, ATP6 and ATP8 (also called A6 and A8) (Mariottini et al 1983).

2.3.2 Genes for translation

All the RNAs needed for mitochondrial translation are encoded by mtDNA in humans and other metazoans. Ribosomal RNAs needed in ribosomes are encoded by 12S and 16S rRNA genes. A set of genes for 22 transfer RNAs is found in human mtDNA. No other tRNAs are known to be imported into mitochondria. Leucine and serine each require two tRNAs, dedicated to different codon groups: Leu(UUR) and Leu(CUN), and Ser(UCN) and Ser(AGY) respectively, whereas all other amino acids each require only one tRNA to read their codons. Extended wobble rules make this possible (Barrell et al 1980).

2.3.3 Other (imported) proteins and RNAs

All the other proteins needed for the respiratory chain, nucleotide translocators, ion transport, the mitochondrial DNA replication and transcription machinery, all proteins of the mitochondrial ribosome and its associated translation factors and, indeed, all other mitochondrial proteins are encoded by the nucleus. These proteins are synthesized in the cytosol and imported into mitochondria through the Tom and Tim complexes (for recent reviews see Schatz 1998, Koehler et al 1999a, Bauer et al 2000), with the help of both cytoplasmic and mitochondrial heat-shock proteins (chaperones).

Recently 5S ribosomal RNA identical to that of cytoplasmic ribosomes was found inside mitochondria, though its function remains to be elucidated (Magalhaes et al 1998). Another RNA, a subunit of RNase MRP, has been shown to be imported into mitochondria in mouse (Chang and Clayton 1989). RNase P is required for generating the mature 5´-ends of tRNAs. It is a ribonucleoprotein having a wide variation in subunit composition between

organisms and organelles (Schon 1999). Only very recently it has been shown to contain a nuclear encoded RNA component in human mitochondria as well (Puranam and Attardi 2001). Mitochondrial ribosomes contain a large number (>50) protein subunits, of which many are unique to mitochondria (Graack et al 1999). Ribosomal proteins are much less conserved in mitochondria than in cytoplasmic (or bacterial) ribosomes (Goldshmidt-Reisin et al 1998). Although in human and other metazoan mitochondria tRNAs are encoded by mtDNA, in some other organisms some or all tRNAs are imported, as in kinetoplastids (Rubio et al 2000).

2.4 Mitochondrial DNA maintenance

2.4.1 Basic mechansims

Cells contain a high copy number of mitochondrial genomes, the number varying from one cell type to another, between 1,000 and 10,000 copies per cell (Larsson and Clayton 1995, Lightowlers et al 1997). Normally short-lived human cells (e.g. sperm, leukocytes) have a low mtDNA copy number, whereas long-lived cells (e.g. skeletal muscle, brain cells, oocytes) tend to have a high copy number of mtDNA. One hypothesis that has been put forward suggests that high copy number may protect mtDNA from the accumulation of a critical threshold of mutations (Chinnery and Samuels 1999).

It is known that in plants (Kanazawa et al 1998) and in yeast (MacAlpine et al 2000) mtDNA can undergo recombination. However, the ability of human mtDNA to undergo recombination has for long been controversial, though evidence supporting various types of recombination seems to be gaining more acceptance (e.g. Holt et al 1997, Awadalla et al 1999, Tang et al 2000a). Recombination at the molecular level, especially intramolecular recombination in somatic cells, seems well documented, but doubts remain about intermolecular recombination. The paper by Awadalla et al, which presented evidence in favour of sexual recombination of mtDNA, has recently been critisized (Jorde and Bamshad 2000, Kivisild and Willems 2000, Kumar et al 2000, Morris and Lightowlers 2000, Parsons and Irwin 2000).

Maintaining a certain mtDNA mass seems to be more important than the copy number itself in human cells (Tang et al 2000b). Earlier it has been shown in yeast (Fukuhara 1969) and also mouse (Howell et al 1984) that cells can titrate their mtDNA content according to the mass, instead of the number of mitochondrial genomes. The regulation of mtDNA copy number is potentially exercised at several steps, including the

initiation of DNA synthesis, where one or more replication factors might be rate-limiting (Moraes et al 1999), the availability and the size of nucleoside pools (Bestwick et al 1982, Lecrenier and Foury, 1995) and the control of dNTP pools (Reichard 1988, Zhao et al 1998). Virtually nothing is known about the physical mechanism that regulates mtDNA copy number despite some knowledge of mtDNA replication mechanisms.

2.4.2 Nucleoid organization

By analogy with eubacteria, as well as the mitochondria of some other eukaryotes, such as slime molds, fungi and plants (Miyakawa 1987, Kuroiwa et al 1998, Kaufman et al 2000), mammalian mitochondrial DNA is believed to be organized in stuctures called nucleoids. This is a protein-DNA complex having 4-5 copies of mtDNA in yeast (Miyakawa et al 1987, 1995) and probably around 5 to tens of copies in humans. The number of nucleoids varies from one organism to another and from one cell type to another (Spelbrink et al 2001). The exact protein composition and organization of nucleoids remains to be elucidated. The transcription factor mtTFA is known to bind mtDNA, therefore it should be located in nucleoids. A putative DNA helicase, related to that of phage T7, also appears to be associated with nucleoids (Spelbrink et al 2001), but surprisingly some other known proteins involved in mtDNA replication, such as DNA polymerase γ, are uniformly distributed inside mitochondria (Spelbrink et al 2000), at least based on reporter assays.

The nucleoid has been proposed to be the unit of inheritance in mitochondria: this is discussed in further detail in relation to the results presented in a later chapter.

2.4.3 Segregation of mtDNA

Normally cells are homoplasmic for a given mtDNA, despite the fact that mtDNA sequence variation between individuals in the population is high, due to a higher frequency of mutations in mtDNA, 5 to 10 times higher than in the nuclear genome (Brown et al 1979, 1982). In accordance with mitochondrial DNA being present in a homoplasmic state in individuals, but nevertheless highly polymorphic in the population, a complete switch of genotypes can be observed within even a single generation. This can be explained by a genetic bottleneck in early oogenesis (Hauswirth and Laipis 1982, Ashley et al 1989, Jenuth et al 1996). Mitochondrial DNA of a mutated genotype may segregate specifically

into cells that will give rise to the germ cells, and thus a shift in genotype can been seen even in one generation, for example in comparing siblings. However, mutations causing disease occur normally in the heteroplasmic state, that is, both wild-type and mutated DNA molecules co-exist in the cell. This may reflect the fact that most disease-causing mutations cannot be tolerated if present in the homoplasmic state, thus leading to early embryonic lethality. Alternatively, maintenance of heteroplasmy during development may involve genetic selection. In either case, only heteroplasmic individuals are ever seen.

Studies of segregation of mtDNA have been carried out mainly using the mouse as a model organism (Smith and Alcivar 1993, Jenuth et al 1996, 1997, Meirelles and Smith 1997). It has been shown that rapid segregation of mtDNA occurs during early oogenesis of mice and is complete by the time the primary oocyte population has differentiated (Jenuth et al 1996). This was demonstrated using heteroplasmic mouse-lines created artificially by micro-injection. The proportion of the donor female mtDNA genotype in the founder was similar to the mean proportion in her offspring, but a shift in individual offspring was observed. No differences were found between mature oocytes and offspring of the given female, indicating that unequal partitioning of mtDNAs into the inner cell mass does not contribute to segregation of mitochondrial genotypes between generations.

It is possible that in human oocytes the biochemical selection against pathological mutations is not strong enough to prevent a certain proportion of them being retained. In dividing cells like blood cells, around a 1% annual decrease in the relative level of a pathological mutation can be detected (Howell et al 2000). At the same time, no change in the heteroplasmy level of a neutral polymorphism was found. However, it was not shown whether individual cells were homo- or heteroplasmic for the mutation. In humans it is possible that, during amplification of mtDNA to its final copy number of over 10^5 in the oocyte, DNA from only one mitochondrion (or perhaps one nucleoid) is preferentially amplified (Blok et al 1997). It is possible also that later in development, only a certain type of mtDNA, i.e. the mutated species becomes preferentially replicated in some cells.

Somatic mutations of mtDNA occurring during human lifespan probably do not contribute meaningfully to phenotype, and in fact may even be hard to detect. Whether such mutations ever segregate to homoplasmy in a healthy individual is not known. Probably the only context in which a mutant mtDNA accumulates within an individual is in clonally derived proliferating cells, i. e. a tumour (Hofhaus and Gattermann 1999).

After mating, budding yeast or even fission yeast reaches the homoplasmic state within a few cell divisions, although the precise mechanisms of this mitotic segregation are not clear. The number of independently segregating units inferred from these studies is about an order of magnitude less than the mtDNA copy number, supporting the idea that at

least in these organisms, the nucleoid is the unit of segregation (Treat and Birky 1980, Thrailkill and Birky 1980).

2.5 Mitochondrial DNA replication

There are currently two models for how mitochondrial DNA is replicated. The first (Clayton 1982), based on a wealth of evidence from electron microscopy and end-mapping studies, is a very unusual mode of replication, being both strand-asynchronous and asymmetrical. There are two replication origins, one for each strand (see Fig 2.2). Synthesis of the leading (H-) strand starts at the heavy-strand origin (O_H) adjacent to the D-loop and continues by strand displacement around two-thirds of the genome. Only after displacing the second origin of replication, O_L, does lagging-strand synthesis begins.

The more recent model (Holt et al 2000) represents more conventional replication. This model proposes that replication starts from a single origin and that the leading and lagging strands are synthesized simultaneously. Both modes of replication can be found in mammalian mitochondria under different conditions. Apparently, if there is a demand for a net increase of mtDNA not linked to the cell cycle, the second mode predominates. The first mode operates in cultured cells without a need for mtDNA amplification. A rapid switch from one replication mode to another can be detected during the recovery phase after experimentally induced depletion of mtDNA (Holt et al 2000).

Thus far, rather little is known about the mitochondrial DNA replication machinery. DNA polymerase γ (POLG) comprises a catalytic and an accessory subunit, the latter responsible for processivity (Lim et al 1999). The catalytic subunit has a C-terminal domain responsible for polymerase activity and an N-terminal domain with 3´-5´ exonuclease activity (Ropp and Copeland 1996, Walker et al 1997). Expression of a mutated version of POLG (carrying a mutation that disables 3´-5´ exonuclease activity) results in the accumulation of mutations in mtDNA (Spelbrink et al 2000). In yeast, overexpression of a variant carrying an inactivating mutation in the polymerase domain results in the loss of mtDNA (Foury and Vanderstraeten 1992). The accessory (β) subunit of polymerase γ (Wang et al 1997, Carrodeguas et al 1999) seems to be involved with the recognition of the RNA-primer in the D-loop (Fan et al 1999, Carrodeguas and Bogenhagen 2000). Another protein involved in replication is the mitochondrial single-stranded DNA binding protein. It is needed to stabilize single-stranded regions of mtDNA (Zeviani et al 1995). The DNA ligase III gene encodes both nuclear and mitochondrial ligase activity (Lakshmipathy and Campbell 1999). Mitochondrial transcription factor A

(mtTFA) binds to both H-strand promoters and to the L-strand promoter (for a discussion of mitochondrial transcription see the following section). In addition to transcriptional activity, this binding is also required for replication, since replication initiation at O_H requires an RNA primer (Clayton 1991). It has been shown, moreover, that loss of mtTFA leads to complete loss of mtDNA and embryonic lethality in mice (Larsson et al 1998). A putative DNA helicase related to that of T-odd phages has recently been inferred to be present in human mitochondria (Spelbrink et al 2001). This protein appears to be located in nucleoids, although its exact role in mtDNA maintenance is unknown.

2.6 Transcription

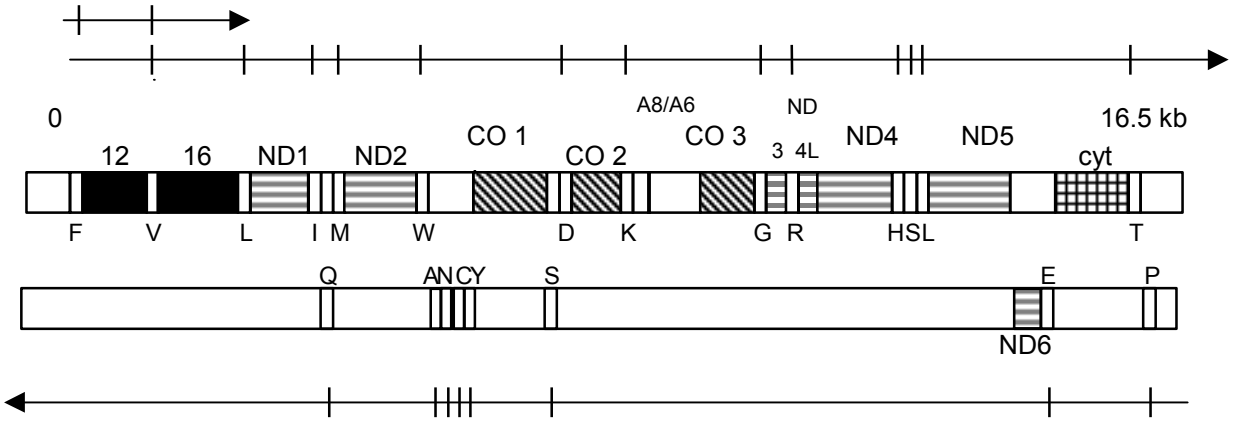


Figure 2.3. Transcription units of mtDNA. Two transcription units of the heavy strand can be found, giving rise to rRNAs or mRNAs. tRNAs (shown as vertical lines on the arrows) separate individual mRNAs, except in the case of ND4/ND4L and A8/A6, which are bicistronic. A single transcription unit is encoded by light-strand. See text and legend to Fig. 2.2 for the names of the genes.

The D-loop is near to the control site for transcription (see review by Clayton 1992), where both heavy and light strand promoters are located (HSP and LSP, respectively). The majority of genes are transcribed from the H-strand. There are two polycistronic transcription units of the heavy-strand (Montoya et al 1983), a 2.7 kb unit containing the ribosomal RNA genes and phenylanine and valine tRNAs, and a 16.5 kb long unit, encompassing both rRNAs, 13 tRNAs (all but phenylalanine) and 10 of the 11 mRNAs. Note that four polypeptides, namely A8+A6 and ND4L+ND4 are translated from bicistronic mRNAs. The remaining 8 tRNAs and one mRNA (ND6) are transcribed from

the L-strand. Both H- and L-strands are transcribed symmetrically in their entirety. mRNAs and rRNAs are separated by tRNAs in most cases, the so-called punctuation model of mitochondrial transcription (Ojala et al 1981). An enzyme to recognize and cleave exactly both 5′- and 3′-termini of tRNAs is essential for this model. RNase P can cut the 5′-end (Doersen et al 1985, Schon 1999) and 3′-processing activity has been found from rat liver (Manam and van Tuyle 1987). In addition, other RNA processing enzymes are needed. In humans there are no untranslated leaders as, for instance, in yeast mitochondrial mRNAs (Fox 1996). Post-transcriptional polyadenylation terminates the rRNA and mRNA sequences and 3′-terminal CCA is added posttranscriptionally to tRNAs. Other RNA modifications occur as well (see Gillham 1994).

Termination of transcription is controlled by mitochondrial termination factor mTERF (Kruse et al 1989). It plays an essential role in the control of the relative transcription of the rRNA and mRNA precursors (Daga et al 1993, Selwood et al 2000). It is a leucine zipper protein that binds DNA as a monomer (Fernandez-Silva et al 1997).

2.7 Translation

The translation of mitochondrial mRNAs occurs inside mitochondria and is separate from cytoplasmic translation. It has several unique features compared to the cytoplasmic or bacterial systems. The ribosomal and transfer RNAs needed for translation are encoded by the mitochondrial genome. Mitochondrial ribosomes contain less RNA and more proteins than bacterial ribosomes (Pietromonaco et al 1991). Mitochondria have a unique genetic code in several aspects. In human mitochondria AUA codes for methionine instead of isoleucine, UGA codes for trypthophan instead of being a stop codon, and AGA and AGG are stop codons instead of coding for arginine (Barrell et al 1979). The wobble rules of Crick (Crick 1966) are relaxed allowing a single tRNA to recognize each of the families of four triplets coding for a single amino acid. Several modifications occur in human mitochondrial tRNAs. The list of modifications and modified nucleosides can be found in Sprinzl et al (1998). In addition, an unidentified modification of uracil at the wobble position in tRNA-Leu(UUR) has been found (Yasukawa et al 2000b). The sequences of mitochondrial tRNAs differ from the common type at a number of positions. The lengths of both the TψC and dihydrouridine (DHU) loops vary considerably in mitochondrial tRNAs. An extreme example is tRNA-Ser(AGY), where the DHU loop is lacking completely. Some mRNAs lack the AUG start codon. Instead AUN and sometimes GUG codons serve as sites for translational initation. Translation starts with fMet-tRNA (Attardi

1985). Also there are differences between the sensitivities to different antibiotics: mitochondrial ribosomes are sensitive to antibiotics known to inhibit bacterial protein synthesis such as chloramphenicol and tetracyclines, but less sensitive to inhibitors of cytoplasmic ribosomes, such as emetine.

In yeast mitochondria 5′ leaders target the mRNAs to specific locations (Costanzo and Fox 1990). In humans the targeting mechanism is not understood, though some mRNA-binding factors are most likely to be involved (O'Brien et al 1990, Denslow et al 1991).

As in bacteria, elongation factor EF-Tu combines with aminoacylated tRNA and GTP to form a ternary complex, which then delivers the tRNA to the acceptor site of the mitochondrial ribosome. Ribosome translocation is catalysed by elongation factor EF-G. Elongation factor EF-Ts regenerates the EF-Tu**.**GTP complex from EF-Tu**.**GDP. Mitochondrial release factor 1 (mRF-1) helps in termination of translation by hydrolyzing the peptidyl-tRNA and releasing the polypeptide from the ribosome (see Gillham 1994).

2.8 Mitochondrial disease

Disease caused by mitochondrial dysfunction can be due to mutations either in the nuclear or mitochondrial genome. Normally the mutations in mtDNA have direct consequences on OXPHOS activity, whereas mutations in nuclear DNA can have indirect effects, for example, primarily affecting mtDNA maintenance or protein import (Jin et al 1996, Tranebjaerg et al 1995, Koehler et al 199b). The effects on mitochondrial function can therefore be secondary. Sometimes drawing the distinction is difficult, for example, in the case of nuclear mutations causing mtDNA rearrangements. The symptoms are caused most likely by mtDNA alterations, but a direct role of the disease mutations in nuclear genes on mitochondrial function or homeostasis can also not be excluded from having a role in pathogenesis.

Mitochondrial DNA mutations cause defects mainly in brain, muscle, heart, vision or hearing, though mtDNA mutations can also be involved in other abnormal cellular states, such as cancer (recently reviewed in Skulachev 1999), diabetes mellitus (recently reviewed in Berdanier 2001), infertility (recently reviewed in Ruiz-Pesini et al 2000) and abnormal cell death (recently reviewed in Desagher and Martinou 2000). Very often these mutations cause severe, multi-system disorders. The other typical feature is a vide variety of symptoms caused by the same mutation in different individuals, although the same phenotype can be caused in different individuals also by different mutations.

The involvement of mtDNA in disease was first discovered by Ian Holt and colleagues (Holt et al 1988) (see section 2.8.1) and Douglas Wallace and colleagues (Wallace et al 1988a, 1988b) (see section 2.8.2). New mitochondrial mutations that cause disease are now being found frequently and their postulated importance in human pathology generally is also increasing. I will discuss several disease-causing mitochondrial mutations as examples of the different types of mutations that can affect mitochondrial function.

2.8.1 Large-scale rearrangements of mtDNA

The very first type of mitochondrial mutations found were large heteroplasmic deletions in patients having various types of mitochondrial myopathies, mainly progressive external ophtaloplegia (PEO, a myopathy affecting only the external eye muscles) (Holt et al 1988) or Kearns-Sayre syndrome (KSS), a multi-system disorder affecting several tissues particularly muscle. Deletions have been found in most (90%) of the patients (Moraes et al 1989). Pearson syndrome is another multi-system disorder, often fatal in early childhood (Rötig et al 1990), which is caused by rearrangements of mtDNA. In Pearson syndrome hematopoiesis, liver, pancreas and kidney are affected. Most cases of the above syndromes are sporadic. These sporadic deletions are most often single primary rearrangements that have occurred early in development and then further propagated clonally to all or many tissues during development. Tissue-distribution plays an important role in patient phenotype. The genes for several tRNAs and mRNAs can be missing from the deleted molecules. Sometimes partially duplicated molecules also can be found (Manfredi et al 1995, Fromenty et al 1997). These can, depending on the site of duplication, at least theoretically encode abnormal proteins which might interfere with mitochondrial function.

2.8.2 Mutations in mRNAs

The first mitochondrial point mutation in an mRNA gene that was discovered causes a maternally inherited disease called Leber's hereditary optic neuroretinopathy (LHON) (Wallace et al 1988a). The G11778A mutation is located in the ND4 gene for a subunit of complex I of the respiratory chain. There is a rapid progressive loss of central vision and atrophy of the optic nerves, usually presenting in late adolescence. There are

several other mutations also known to cause LHON, mainly in other genes for complex I, including ND1, ND4 and ND6. Surprisingly there is no muscle phenotype. These mutations seem to affect males more often than females, and in addition, the mutation can be found in the homoplasmic as well as the heteroplasmic state. These peculiar features of the disease are still not understood. The heteroplasmic T8993G mutation in the A6 gene causes neurogenic muscle weakness, ataxia and retinitis pigmentosa (NARP) (Holt et al 1990). The mutation is believed to alter the assembly of complex V (Nijtmans et al 1995, 2001). When the mutation load is at least 90%, the mutation leads to the more severe and usually fatal condition designated as Leigh syndrome (Tatuch et al 1992). This occurs normally in early infancy and patients suffer from lesions in the brainstem and spinal cord, lactic acidosis and failure to thrive (Rahman et al 1996). It is surprising that this mutation affects mainly the central nervous system, and not the muscle as one might expect. The missense T9957C mutation in COXIII converts a highly conserved phenylalanine to leucine and is one of the mutations associated with the MELAS syndrome (see below) (Mandfredi et al 1995). Another point mutation in ND5 can cause a MELAS as well (Corona et al 2001). Disease causing point mutations can sometimes be found in healthy maternal relatives but have otherwise not been found in the normal population.

2.8.3 Mutations in rRNA genes

A point mutation at np 1555 in the 12S ribosomal RNA gene is found in families with maternally inherited non-syndromic sensoneuronal deafness (Prezant et al 1993), as well as aminoglycoside-induced deafness. This is the only mitochondrial rRNA mutation shown to be involved in human disease so far. There are some other, putatively pathological rRNA mutations found in humans (e. g. Thyagarajan et al 2000), but cybrid analysis and further description of pathological phenotypes will be needed to demonstrate their role in disease.

2.8.4 Mutations in tRNA genes

Mutations in any of the 22 tRNAs encoded by mtDNA can potentially cause a severe defect in protein synthesis, thus having an effect on all protein subunits encoded by mtDNA. This is the reason why patients having tRNA mutations very often suffer from a variety of symptoms and have several tissues affected. On the other hand, there are also

some tRNA mutations associated with very mild, tissue-restricted disorders. One of the most studied disorders associated with a mitochondrial tRNA point mutation is myoclonic epilepsy associated with ragged-red fibers (MERRF), caused by the A8344G mutation in tRNA-Lys (Shoffner et al 1990). Mutant cells have 50-60% decrease in tRNA(Lys) aminoacylation. In addition, premature termination of translation takes place at or near each lysine codon (Enriquez et al 1995). This mutation can be tolerated to rather high levels: unaffected relatives can have as high as 70% of mutated DNA (Hammans et al 1993).

The other extensively studied disease group is that associated with mutations in the gene for tRNA-Leu(UUR). This gene seems to be a hotspot for mutations, in the sense that many different pathological mutations have been found in it. The pathological phenotype associated with these mutations is extremely variable, even in the presence of the same point mutation between different families or individuals. Mutations found in tRNA-Leu(UUR) and the diseases caused by it are listed below:

Mutation	**Clinical phenotype**	**Reference**
A3243G	MELAS	Goto et al 1990, Kobayashi et al 1990
	diabetes and deafness	van den Ouwenland 1992
	PEO	Moraes et al 1993
	diabetes	Janssen et al 1999
A3251G	myopathy	Sweeney et al 1993
A3252G	encephalopathy	Morten et al 1993
C3256T	myoclonic epilepsy	Moraes et al 1993, Sato et al 1994
	and RRF/ocular myopathy or MELAS	
A3260G	myopathy and cardiomyopathy	Zeviani et al 1991
T3271C	MELAS	Goto et al 1991
T3291C	MELAS	Goto et al 1994
A3302G	myopathy	Bindoff et al 1993
C3303T	myopathy and cardiomyopathy	Silvestri et al 1994
T3272 del	encephalopathy	Shoffer et al 1995

Table 2.1. Mutations of tRNA-Leu(UUR).

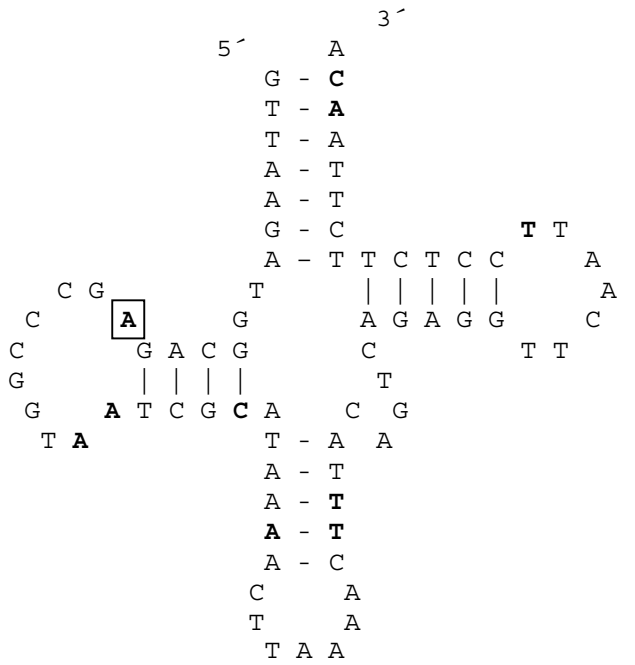


Figure 2.4. The DNA sequence of mitochondrial tRNA-Leu(UUR). The nucleotides affected by the most common mutations found are shown in bold, and in addition, the most common A3243G mutation is in the box. See also table 2.1.

The most common mutation, A3243G, is located within the conserved binding site for mTERF (Hess et al 1991) and the mutation disturbs the binding of the protein *in vitro*. Complex I seems especially to be affected (Goto et al 1992, Morgan-Hughes et al 1995), which might be due to the fact that 8% of the amino-acids of ND6 are, unusually for mitochondrial genes, encoded by UUR codons. See more details in section 2.9.

Only a few mutations to date have been found in the gene coding for the other leucine tRNA, reading the CUN codon group. The first to be found, at np 12311 mutation causes chronic PEO in the Japanese population (Hattori et al 1994). The G12315A mutation is associated with mitochondrial encephalomyopathy (Fu et al 1996). Isolated skeletal myopathy is found to be associated with the A12320G mutation (Weber et al 1997). As an example of how differences in a single tRNA can cause different disorders, polymorphic A12308G is associated with an increased risk of strokes (Pulkes et al 2000). In addition, mutations are found in many other mitochondrial tRNA genes, for example, in the tRNA-Ser(UCN) gene causing hearing loss, ataxia and myoclonus (Tiranti et al 1995) or in tRNA-Cys associated with mitochondrial encephalopathy (Manfredi et al 1996).

Typical for almost all mutations in mtDNA is the so-called threshold effect. That means that only when the mutation is at a sufficient relative amount in the cell is a clear defect caused. This applies to point mutations as well as deletions or partial duplications. The exact threshold level varies from one mutation to another, and also there are differences between families even for a given mutation (e.g. see Moraes et al 1993, Matthews 1995, Chinnery 1999). Tissue-distribution also plays an important role in the variability of symptoms between individuals (i.e. Hurko et al 1990, Weber et al 1997).

2.9 Cybrid methodology

Cybrid cells have enabled us to study mitochondrial mutations in a more controlled fashion. This methodology was extensively described by King and Attardi (1989) and involves fusions between two different kinds of cells. One cell line, the recipient, has previously been treated with ethidium bromide to eliminate mtDNA completely. These rho^0 cells thus cannot respire and are dependent on uridine and pyryvate, but of course, do have mitochondria. Other cells, i.e. from a patient, are enucleated, giving cytoplasmic fragments (cytoplasts) containing mtDNA. When these cytoplasts and rho^0 cells are fused, a series of cybrid cell lines are created that contain the same nucleus but different kinds or proportions of mutant mtDNA (King and Attardi 1989, 1996).

Cybrid cells have made it possible to follow the cellular consequences of a specific defect caused by a single mutation (see examples in King et al 1992, Koga et al 1995, Raha 1999) and in addition, it is possible to show that the pathology is due to the mtDNA instead of nuclear DNA (as in Mariotti et al 1994). In studies of segregation of mtDNA, cybrids have also been a very powerful tool (Matthews et al 1995, Fu et al 1996). Moreover, using rho^0 cells it is possible to monitor changes in nuclear gene expression associated with decreased respiration (Li et al 1995).

In addition, the cybrid method has recently been used to investigate evolutionary aspects of mtDNA. So called xenomitochondrial cybrids, where nucleus and cytoplasm originate from different species, have given deeper understanding about evolutionary paths of closely related species, such as humans and apes, or mice and rats, as well as to fill in gaps in knowledge concerning the interactions between the two genomes of the cell (Barrientos et al 1998, Yamaoka et al 2000).

Cybrid cells have been extensively used in studies of the pathological phenotype caused by the A3243G mutation, with the aim of explaining the wide variety of symptoms caused by this mutation. Osteosarcoma cell cybrids with high levels of A3243G mutant

mtDNA show defects in mitochondrial protein synthesis and respiration (Chomyn et al 1992, King et al 1992, Dunbar et al 1996), confirming the pathological nature of the mutation. Defects in RNA processing are also found, since the accumulation of RNA 19 (spanning 16S rRNA + tRNA-Leu(UUR) + ND 1) can be detected (Schon et al 1992, Koga 1995). The amount of the tRNA itself, its stability, aminoacylation and base-modification are all decreased (see original papers I, II and chapter 5, plus recent reports by Helm et al 1999, Yasukawa et al 2000b). Any of these may contribute to impaired protein synthesis in cybrid cells. A tRNA-Leu(UUR) aminoacylation defect associated with the A3243G mutation has recently been detected also *in vivo*, in patient-derived muscle biopsies (Börner et al 2000). Segregation of the A3243G mutation (shift or resolution of heteroplasmy) has also been studied using the cybrid method (Yoneda et al 1992, Dunbar et al 1995, Shoubridge et al 1995, Matthews et al 1995, Bentlage 1996), providing some insights into possible mechanisms influencing the distribution of the amounts of mutated mtDNA in different tissues. In particular, the direction of the shift in genotype can be different, depending on the parental cell line (Dunbar et al 1995).

3. Aims of the study

These experiments were designed to determine the precise cellular effects of one particular disease-associated mitochondrial tRNA and establish how the defect can be suppressed. An additional aim of the study was to investigate how a heteroplasmic mutation is segregated during cell division in a cell-culture model. This also has major implications for understanding the phenotypes associated with heteroplasmic mtDNA disease.

More specifically, the aims of the study were:

1) to determine the molecular effects of the A3243G mutation in a cell-culture (cybrid) model

2) to test out strategies for suppressing the effects of this mutation and to elucidate the molecular mechanism of suppression

3) to determine the parameters of segregation of mtDNA heteroplasmy in human lung carcinoma cells

4) to identify nuclear factors that influence heteroplasmic segregation in these cells

In the course of the preliminary studies, a spontaneous suppressor mutation was discovered, which provided the ideal material to address all four questions.

4. Materials and methods

The materials and methods are described in detail in the original publications I-III and V. General principles and specific methods relating to the unpublished experiments are summarized here.

4.1 Cell lines and culture

Human A549 lung carcinoma and 143B osteosarcoma cybrid cell lines containing A3243G mutation were originally created in the laboratory of Ian Holt (Dunbar et al 1995). The cytoplasmic donor was a patient suffering MELAS syndrome. The following A549 lung carcinoma-derived cell-lines were used in the initial study (%A3243G mutant mtDNA shown in parentheses): B (0%), Y (81%), X (95%) and G (99%). The various derivatives from these lines that were isolated in the course of the study are described in Results. Additional (serial) cybrids, derived by fusion of cytoplasts from the suppressor line G_T with A549 and 143B rho^0 lines carrying nuclear selectable markers, were obtained from Nicole Hance, University of Dundee. Details of their derivation are reported in Hance's doctoral thesis (2001). The genotypic and phenotypic characterization of these serial cybrid cell-lines is presented in Results, chapter 5.1.

The cells were routinely passaged weekly. The medium used was high-glucose DMEM (Biochrom) (4.5 g/l of D-glucose), supplemented with 10% FCS (Gibco), 2 mM L-Glutamine (Bio-Whittaker), 100 U/l Pen-Strep (Bio-Whittaker), 1 mM sodium pyruvate (Amersco) and 50 μg/ml uridine (Sigma). Cells were trypsinized after washing with PBS. 1 ml of trypsin (500 mg/l) (Bio-Whittaker) was added to a 100 mm plate and then removed immediately. Plates were further incubated 2-4 minutes at RT. After that a tiny amount of fresh medium was added and cells were either re-seeded on a new plate or else collected by centrifugation. Cell cloning was performed using serial dilutions on 96 well plates. Cell clones were collected from rows that contained many fewer cell colonies than wells. In addition, microscopy, carried out soon after the cells were attached (normally a few hours), was used to exclude any wells that clearly contained more than one cell.

4.2 Mitochondrial genotyping

The amount of heteroplasmy for the A3243G and G12300A mutations was measured in the following way: DNA was extracted from cells by detergent lysis and proteinase K digestion (20 mg/ml) as described previously (Reid et al 1994, 1997). The regions around the mutation sites were PCR-amplified using mtDNA-specific primer pairs, with addition of 1 µl of 1:10 dilution of [α-^{32}P]-dCTP in the last cycle of PCR (in total 23-24 cycles), with annealing temperature 60 ºC. PCR conditions are given in detail in article I. Labeled PCR products were then digested with the restriction enzymes diagnostic for the site polymorphisms created by the mutations (*Apa*I and *Afl*II, respectively, for A3243G and G12300A), and products of replicate digestions were analysed by 4% native polyacrylamide gel electrophoresis and phosphorimaging. Originally 8 replicates of each sample were used. For later experiments, once the reliability of the method was clear, the number of replicates was decreased to four.

4.3 Southern blots

For Southern blot analyses of relative mtDNA copy number, *Eco*RI-digested genomic DNA was prepared from cells as follows. One confluent 100 mm plate (approximately 5 x 10^6 cells) was trypsinized, and cells recovered by centrifugation for 3 min at 2,000 g_{max}. Cell pellets were resuspended in 50 µl of water, 5 µl of 10 mg/ml boiled RNase A were added, and samples were incubated for 30 min at 37 ºC. After addition of 10 µl of Proteinase K solution (18 mg/ml) and 350 µl of 6 M guanidine hydrochloride, 0.5 M sodium acetate pH 5.5, incubation was continued overnight at 56 ºC. Crude DNA was precipitated by the addition of 800 µl of ice-cold isopropanol, and samples were stored at 4 ºC before further processing (overnight to 3 weeks). Crude DNA was recovered by centrifugation for 30 min at 7,000 g_{max}, and pellets, washed twice with 70% ethanol, were digested three times successively with *Eco*RI as follows. Pellets were resuspended by the successive addition of 89 µl of water, 10 µl of 10 x *Eco*RI digestion buffer (New England Biolabs) and 1 µl of *Eco*RI (20 u/µl, New England Biolabs) and were incubated overnight at 37 ºC. In the first digestion cycle an additional 1 µl of the enzyme was added after 5 hours. Samples were then phenol-chloroform extracted without vortexing, centrifuged at 7,000 g to separate phases, and ethanol precipitated. The thrice-digested DNA was finally resuspended in 30 µl of water.

DNA prepared as above was fractionated on a 0.7% agarose gel, capillary-blotted under standard conditions to MagnaCharge nylon membrane (Micron Separation Inc., Westborough, USA) and u.v.-linked. Blots were hybridized overnight at 65 ºC in 1 mM EDTA, 7% SDS, 0.5 M sodium phosphate buffer pH 7.2 with radiolabeled probes for human mtDNA (a PCR product derived from a cloned segment of the COXII gene) and rDNA, as described by Spelbrink et al (1997). The rDNA probe was a PCR product for the first 500 bp of the 18S rRNA gene, obtained by amplification from genomic DNA of a control A549 cybrid (line B) using primers 18S-F (TACCTGGTTGATCCTGCCAG) and 18S-R (TCGGGAGTGGGTAATTTGC). Amplification conditions were as for mtDNA genotyping (annealing temperature 60 ºC) and probe fragments were purified using the QIAquick PCR purification kit (Qiagen), and labeled using the Oligolabelling Kit (Pharmacia Biotech) in the presence of [α-^{32}P] dCTP (Amersham, 3000 Ci/mmol). After hybridization, filters were washed successively for 20 minutes each at 65 ºC in 3 x SSC, 0.1% SDS and 0.3 x SSC, 0.1% SDS, then analysed by autoradiography and quantitated using a Molecular Dynamics PhosphorImager SI™ with Image-Quant™ software.

4.4 Northern blots

Total RNA was extracted from growing cells using TRIzol™ Reagent (Life Technologies), under the manufacturer's recommended conditions. RNA was fractionated either on formaldehyde/1.2%agarose gels (I) or 7 M urea-6.5% polyacrylamide gels (II). In the former case, RNA was blotted onto Hybond™-Nylon membrane (Amersham) and in the latter onto Zeta-Probe GT membrane (Bio-Rad). Oligonucleotide probes were labelled using [γ-^{32}P] ATP (Amersham, 3000 Ci/mmol) and T4 polynucleotide kinase (New England Biolabs). The probes used were:

Leu(UUR): GTTTTATGCGATTACCGGGC, corresponding with np 3263-3244 of the human mtDNA sequence (Anderson et al 1981).

Leu(CUN): TGCACCAAAATTTTTGGGGC, corresponding with np 12320-12301 of the human mtDNA sequence.

Hybridization was carried out overnight at 42 ºC in 50 mM Pipes, 100 mM NaCl, 50 mM sodium phosphate, 1 mM EDTA, 5% SDS, pH 6.8. Blots were washed twice at

room temperature (RT) for 5 minutes with 2 x SSC, 0,1% SDS and then 10-20 minutes at 42 ºC in 1 x SSC, 0.1% SDS. Blots were visualized by autoradiography and/or quantitated for signal by phosphorimaging. Filters were stripped either twice for 20 minutes in 1 x SSC, 0.1% SDS at 95 ºC (II) or in boiling 0.5% SDS and then left to cool to RT.

4.5 Protein gel electrophoresis

4.5.1 Two-dimensional electrophoresis

For 2-D polyacrylamide gel analysis about 5 million cells were collected, washed in PBS and resuspended in 100 µl of PBS. One hundred µl of digitonin (2 mg/ml in PBS) was added, and the sample was incubated for 15 min on ice and then diluted 7-fold with ice-cold PBS and microcentrifuged for 5 min at 4°C, at 13,000 g_{max} . After washing twice with PBS the pellet was resuspended in 50 µl gel buffer (1.5 M aminocaproic acid, 150 mM Bis-Tris, pH 7.0) and 50 µl of 2 M aminocaproic acid and 20 µl 10% (w/v) lauryl maltoside were added. After centrifugation for 30 min at 4°C, 16 000 g_{max}, 10-50 µl of the the supernatant was loaded onto the gel. The first dimension used a gradient blue-native gel, the second dimension an SDS gel, both made according to Schägger and von Jagow (1991).

4.5.2 Analysis of mitochondrial translation products by SDS-PAGE

Cells were grown to 70% confluence on a 60 mm plate, washed once with DMEM lacking methionine and then incubated in this medium for 10 min at 37 °C. After washing, cells were incubated for 15 min at 37 °C in DMEM lacking methionine/10% FCS/2 mM L-glutamine plus 10 µg/ml emetine (ICN Biochemicals Inc.) to block cytosolic translation. Then ^{35}S methionine (1000 Ci/mmol, Amersham Pharmacia Biotech) was added to a final concentration of 20 µCi/ml and cells were incubated for 30 min at 37 °C. After incubation labelled cells were washed twice with DMEM and trypsinized as usual. Cells were suspended in 67.5 µl PBS, 7.5µl 15% lauryl maltoside and 1 µl of PMSF and then incubated 30 min on ice. After 1-5 min of micro-centrifugation the supernatant was collected and 100-200 µg of total protein was loaded and run on a 12% polyacrylamide gel (Schägger and von Jagow 1991).

4.6 *In situ* cytochemistry

For staining by the mitochondrial redox-sensitive dye JC-I (see article III), cells were grown almost to confluence on 60 mm plates, washed with PBS, then stained for 15 min at 37 ºC in 1 ml of serum-free Optimem medium (Gibco) containing 10 µg/ml JC-1 (5,5´,6,6´-tetrachloro-1,1´,3,3´tetraethylbenzinidezolylcarbocyanine iodide; Molecular Probes). After incubation the cells were washed twice with Optimem, then twice with PBS, mounted in PBS containing 5% sucrose and examined within 20 min under highest magnification on an Olympus BX-50 fluorescence microscope, using a U-MWB filter (III).

For staining by Mitotracker Red (Molecular Probes, see article V), the dye stock at 1 mM in DMSO was diluted 1:10000 in DMEM (i.e. to 100 nM). Cells were incubated 15 min at 37 °C, washed twice with PBS and incubated 2 hours in normal DMEM. Cells were then washed twice with PBS and fixed for 15 min in 4% formaldehyde/5% sucrose, washed twice with PBS and mounted with vecta-shield (Vector Laboratories Inc).

To visualize mitochondrial nucleoids, cells were transfected with an EGFP (enhanced green fluorescent protein) reporter construct for the nucleoid localized protein Twinkle (Spelbrink et al 2001, see article V). Transfection used 10 µl lipofectamine reagent (Gibco) + 900 µl Optimem, which was added dropwise to a solution of 1 µg plasmid DNA plus 100 µl Optimem.

4.7 CGH and FISH

For comparative genomic hybridization (CGH) genomic DNA (proteinase K-extracted, stored frozen) was RNase treated, then deproteinized by a standard phenol-chloroform extraction. 4 µg aliquots of DNA were nick translated in the presence of either TexasRed-6-dUTP or FITC-12-dUTP (DuPont) to provide the respective samples for comparison. CGH analysis was performed as described previously (Kallioniemi et al 1994, Karhu et al 1997).

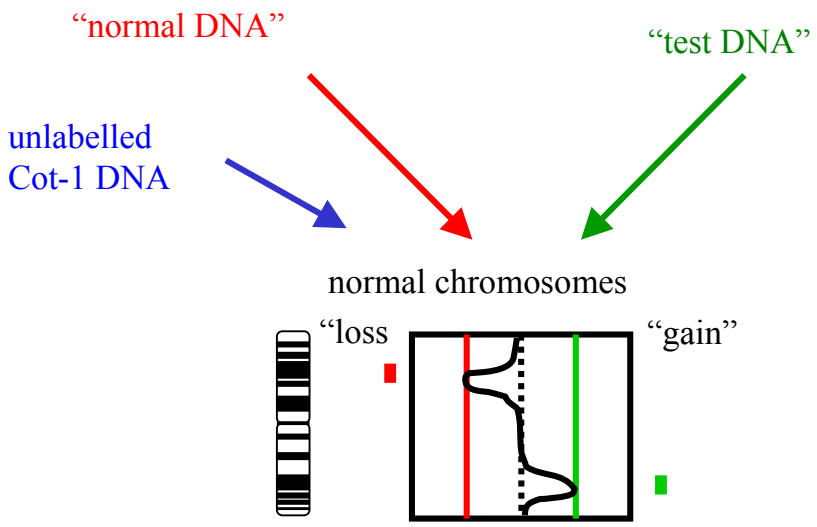


Figure 4.1. The principle of CGH (comparative genomic hybridization). CGH was originally developed to detect amplifications and losses in human chromosomes in cancers. The normal DNA does not contain any losses or amplifications, whereas the test DNA comes from a cancer tissue. I used DNA from line G_T as "normal" DNA and DNA from line G_{TS} as "test" DNA.

For FISH (fluorescence *in situ* hybridization) analysis cells from over-confluent plates were isolated by trypsinization (cells were kept on the plate approximately 3 days after reaching confluence without any medium change). The cell pellet was resuspended in 5 ml 75 mM KCl, and incubated for 20 min at 37 ºC. After re-centrifugation 1 ml freshly mixed 3:1 methanol/acetic acid was added while vortexing. This was further repeated twice. After the final centrifugation cells were suspended in 0.5 ml of the fixative and dropped onto ethanol-cleaned slides. Before hybridization, slides were denatured for 2 min in 70% formamide, 2 x SSC at 68 ºC, dehydrated in ethanol and air dried at 37 ºC. Hybridization was carried out as described previously by Kallioniemi et al 1992 using centromere 12 probe (pA12H8 in pBS, American Type Cell Culture, ATCC) and centromere 9 (q12) probe (pHuR98 in pBR322, ATCC). Probes were nick-translated using direct labels, FITC-12-dUTP (DuPont) for cen 12 and TexasRed-5-dUTP (DuPont) for 9q12.

4.8 Computer modeling of mitotic segregation

Computer prediction of changes with time in the distribution of mtDNA heteroplasmy values in the cell populations studied in the laboratory was performed by K. M. I. Juhola and M. K. Juhola, using a customized computer programme (see article III). The programme was based on a simple mathematical model modified from that used by Hoppensteadt and Peskin (1991) to predict the segregation of plasmids in bacteria.

4.9 Whole-cell oxygen consumption

For whole-cell oxygen consumption assays a near confluent plate was trypsinized normally. Cells were resuspended in glucose-free RPMI 1640 medium (Gibco-BRL) to a final concentration 5 x 10^6 cells/ml. The rate of oxygen consumption was then measured in an aliquot of this suspension (minimum 300 µl), using a HansaTech O_2 electrode with control box CB1D.

5. Results

Results are described in detail in the original publications I-III and V, and summarized here. Unpublished results are shown below.

5.1 Respiratory phenotype of A3243G cybrids (I)

[Note that article I contains a mistake: the whole cell O_2 consumption values shown in Table 1 should be divided by 2.]

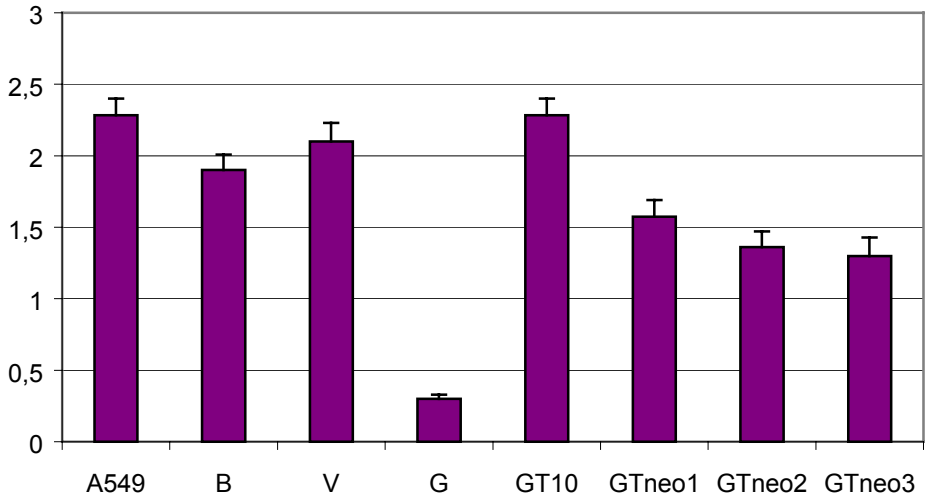
The respiratory phenotype of A549 lung carcinoma cybrid cells containing the A3243G mutation was investigated via studies of (a) whole cell respiration, measured using an oxygen electrode, (b) the synthesis of mitochondrial translation products, by pulse-labeling, and (c) the assembly status of the mitochondrial respiratory chain complexes, by means of two-dimensional polyacrylamide gel electrophoresis. The cybrid line (line G) with 99% mutant mtDNA showed very impaired mitochondrial function by all these parameters. Surprisingly, I identified a derivative of this line, designated G_T, still carrying 99% A3243G mutant mtDNA, but with normal respiration, similar to that of control cybrid cells containing 0% A3243G mutant mtDNA. In addition, mitochondrial protein synthesis and protein complex assembly were normal in this suppressor line. Further analysis showed that cell line G_T was heteroplasmic for an additional mtDNA mutation, the point mutation G12300A in the anticodon of tRNA-Leu(CUN) (I).

5.2 Mitochondrial inheritance of the suppressor

In order to confirm by a rigorous genetic test that the suppressor was indeed mitochondrial, serial cybridization was carried out. Serial cybrids were created by Nicole Hance, University of Dundee, and sent to me for further analysis. Both 143B osteosarcoma- and A549 lung carcinoma-derived rho^0 cells were used as nuclear recipients, in order to determine if the suppression is dependent on nuclear background. Clones with different levels of the suppressor mutation were isolated, with the intention to confirm that the suppression is due specifically to G12300A and not some other mtDNA

marker. In the osteosarcoma nuclear background the protein synthesis phenotype of 100% A3243G mutated cells was not easily distinguishable from the wild-type cells (data not shown), therefore whole cell respiration was used as the most sensitive marker of impaired mitochondrial function. The serial cybrids in the lung carcinoma background (G_T neo clones, so designated because the nuclear donor carried G418 resistance as a selectable marker), were >99% mutant at np 3243, but contained variable amounts of the G12300A suppressor mutation. The selection was withdrawn after cell clone isolation. Their respiration was close to the range seen in some wild-type cells, but less than that of the original G_T line or control cybrids studied alongside (Fig. 5.1). In the osteosarcoma background (Fig. 5.2) the suppression appeared to be significantly less, and was worst in the line containing the highest level of the G12300A mutation.

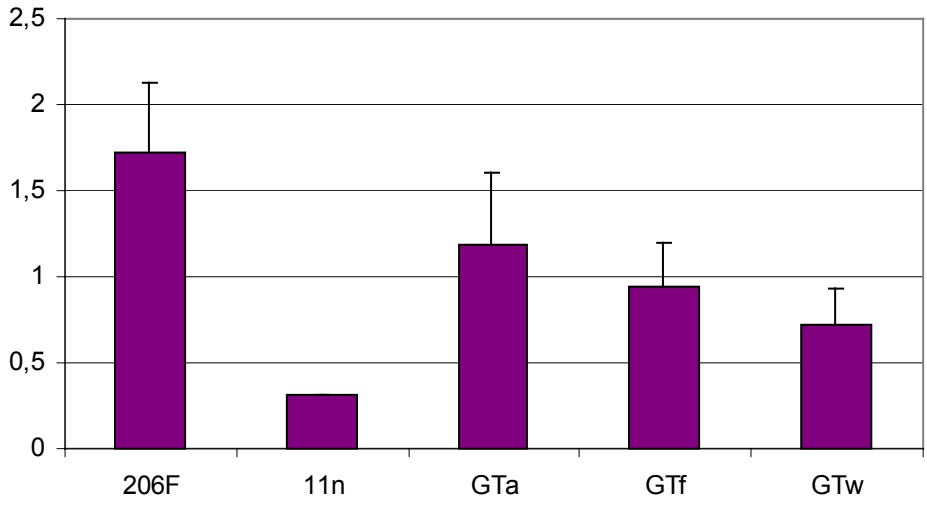
Oxygen consumption
fmol/cell/min



A3243G	0%	0%	0%	99%	99%	99%	99%	99%
G12300A	0%	0%	0%	0%	56%	15%	28%	23%

Figure 5.1. Respiratory phenotype of A549 lung carcinoma serial cybrids. The A549 cell line is the parental cell line of the *rho-zero* recipient used to create the cybrids. Both B and V are wild-type control cybrids and G is the 99% MELAS A3243G-mutated line without the suppressor mutation. Line GT10 (G_{TS}-10 in article III) originated from a shifted, and then stabilized cell clone (see article III). G_{Tneo} lines are serial cybrids having the original G_T line as a cytoplasmic donor. On vertical axis is shown the oxygen consumption as fmol of oxygen consumed by a cell in a minute. Respiration of each cell line was measured three times.

Oxygen consumption
fmol/cell/min



A3243G	0%	100%	100%	100%	100%
G12300A	0%	0%	13%	7%	34%

Figure 5.2. Respiration phenotype of 143B osteosarcoma serial cybrids. Cell line 206F is a wild-type control cybrid line, 11n a 100% A3243G-mutated line without the suppressor mutation. G_{Ta}, G_{Tf} and G_{Tw} are serial cybrids having the original G_T line as cytoplasmic donor. Respiration of line 11n was measured once, all the others are means of three measurements.

The G12300A mutation was earlier shown to be present in mitochondrial DNA instead of being a nuclear pseudogene (I) based on semi-quantitative PCR using mitochondrially enriched samples. However, its ability to restore the pathological phenotype caused by the A3243G mutation appeared to be at least partly nuclear background dependent: it worked better in lung carcinoma cells than in osteosarcoma cells. It is worth noting, however, that lung carcinoma serial cybrids $G_{Tneo1\text{-}3}$ showed a similar proportional decrease in respiration compared to control cell lines as did the osteosarcoma serial cybrids. This may indicate that suppression is not complete in either background, and suggests that the nuclear genotype or expression profile of the original suppressor line G_T may have played some role in the effectiveness of suppression. On the other hand, the copy number of mtDNA of these cell lines was not measured, therefore it is possible that the lesser suppression capacity was due to a reduced amount of mtDNA.

5.3 The G12300A mutation *in vivo* (I)

In order to find out if the original patient or healthy maternal relatives or any other patients affected by the A3243G mutation also contained the suppressor mutation, DNA from several, coded blood samples was screened for the G12300A mutation. None of the affected A3242G patients, nor their healthy maternal relatives, nor the original patient who had been the cytoplast donor had the suppressor mutation. It seems that this suppressor mutation has arisen spontaneously in cell culture, and having selective advantage over the original G cell line, has taken over the culture.

5.4 Effect of the A3243G mutation on protein synthesis (I, II)

Further studies of the A3243G mutant phenotype showed that tRNA-Leu(UUR), the tRNA in which the mutation lies, was present at much lower abundance in cells containing high levels of the A3243G mutation than in control cybrid cells (I). In addition, there was a clear defect in aminoacylation of the mutant tRNA (II). However, the putative suppressor tRNA (i.e. the tRNA containing the G12300A suppressor mutation) was found to be aminoacylated normally (II) and the amount of tRNA-Leu(CUN) was even slightly increased in suppressor cells compared to control cells (I). In addition, two isoforms of tRNA-Leu(CUN) were found in several of the cell-lines studied, although the ratios of the two isoforms varied from one cell line to another, and appeared to respond to the presence of the A3243G mutation.

Both qualitative and quantitative changes in the pattern of mitochondrial protein synthesis were seen in A549 cells containing 99% A3243G mutant mtDNA, whereas 99% mutant cells with the suppressor mutation showed normal mitochondrial protein synthesis (Fig. 5.4). Three abnormal translation products synthesized in 99% mutant cybrid line G, are marked as p, q, r in Fig. 5.4. On the other hand, there were no clear qualitative or quantitative differences in mitochondrial translation in 143B osterosarcoma cells containing 100% A3243G mutant mtDNA compared with control cells (data not shown).

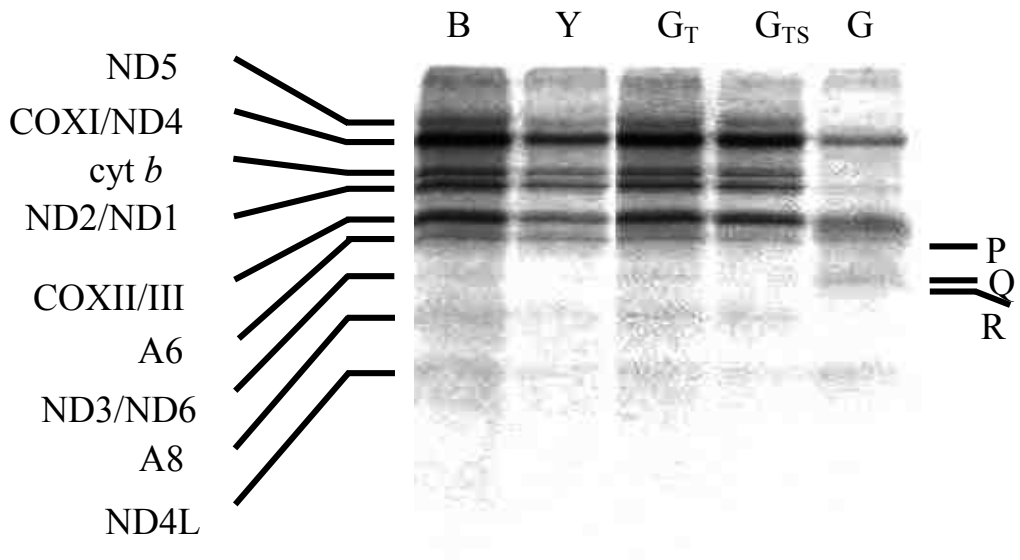


Figure 5.4. Mitochondrial translation in lung carcinoma cybrid cell lines. Line B is the wild-type control cell line and line Y contains 81% A3243G mutant mtDNA. Lines G_T, G_{TS} and G all contain 99% A3243G mutant mtDNA and in addition line G_T contains 11% and line G_{TS} 29% of the suppressor G12300A mutation. Identification of protein subunits is based on a combination of the literature (Spelbrink et al 1994) and immuno-precipitation experiments (data not shown).

Cells containing a high percentage (>70%) of mtDNA with the suppressor mutation, and which were still mutant at np 3243 were derived in the course of long-term culture experiments described in article III. Even these showed a normal mitochondrial protein synthesis pattern. In Figure 5.5. cell clones containing different amounts of the suppressor mutation are shown as an example. These cell lines are cell clones derived from line G_{TS} (see articles III, V). All the cells are 99% for the A3243G mutation.

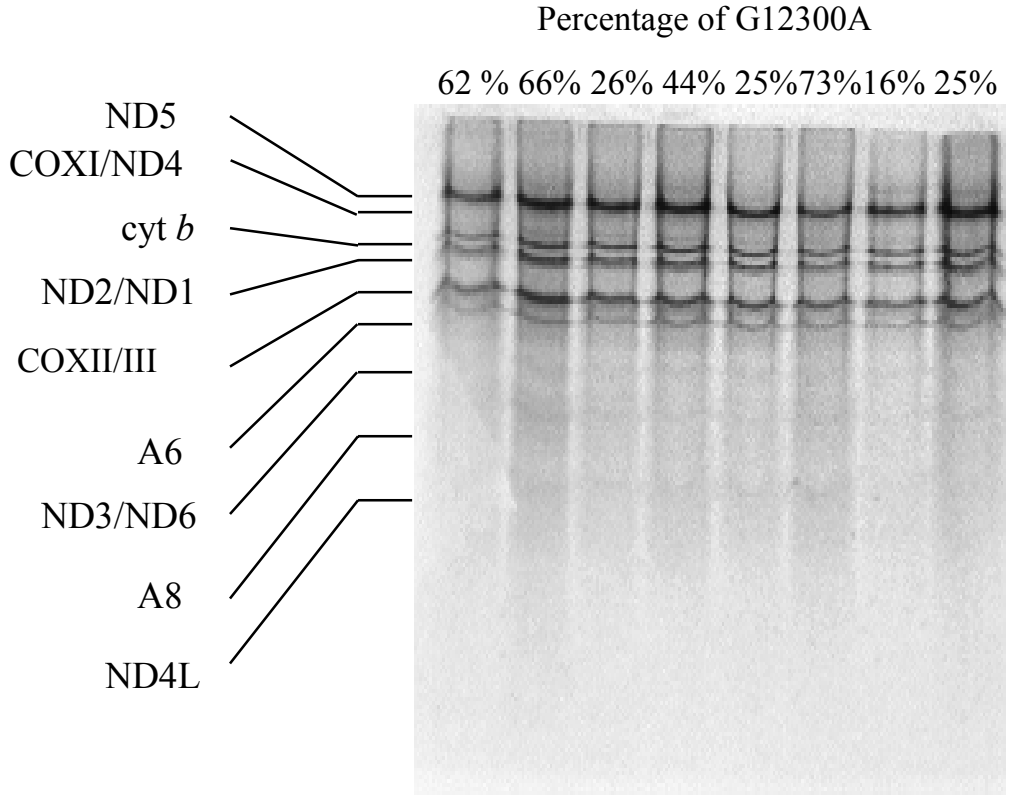


Figure 5.5. Mitochondrial translation studies of individual cell clones having different heteroplasmy level of G12300A.

5.5 Long-term maintenance of heteroplasmy (III)

In a heteroplasmic disease the mtDNA haplotype and even the nuclear background are unlikely to be the sole determinants of phenotype, because of the fact that heteroplasmy may not be stable, and that any instability may itself be influenced by tissue-specific background effects. Nuclear background is clearly of significance in determining the direction of heteroplasmic segregation for A3243G (Dunbar et al 1995), but it leaves unanswered the question of how fast and how far such segregation actually goes. The availability of a panel of cybrids lines, some of which also contained the suppressor mutation, provided a context in which it was possible to address these questions.

Cell lines Y, X and G_T were continuously cultured for long periods of time (3 months to 2 years). During this time they showed remarkable stability of mitochondrial genotype. Average heteroplasmy levels remained unchanged, and heteroplasmy levels measured in individual cell clones after such periods showed a very low diversity (article III). Based on mathematical modelling carried out by MK Juhola and KMI Juhola, I

estimated the segregation number (the number of independently segregating genetic units) as being > 1000 (III).

Even though line G grew normally in bulk culture, it was unclonable using the method used for these experiments, therefore it was not possible to estimate segregation number in this line that had very high levels of A3243G mutant mtDNA. However, line G contained a small number of cells having 5-10% of wild-type DNA. If cells were grown on galactose-medium without glucose, all the cells except about 1 in 300,000 died. All these surviving cells were shown to have only 90-95% of the A3243G mutation. Therefore, very slow mitotic segregation is inferred to be taking place in this cell line as well.

Overall, the cell culture model suggests that heteroplasmic segregation is constrained in some way. In article IV (and below, in Discussion) I present in detail a model for mtDNA organization in the cell that can account for these and other observations I shall now describe.

5.6 Heteroplasmic shift and restabilization (III)

In the course of these studies, another unexpected observation was made. After recloning cells from a sub-culture of line G_T for segregation analysis as referred to in the previous section, a completely different heteroplasmy pattern was found, compared with the one observed initially in the same cell-line. In this sub-culture, now designated G_{TS}, there was a wide distribution of heteroplasmy levels between cell clones. In addition, many of these clones shifted further in heteroplasmy level before reaching a stable mtDNA genotype, in each case within a period of several months (III, Figures 3 and 4). In a follow-up experiment, each clone was recovered from stock frozen down at an early time point during this genotypic shift, and re-grown for the same time periods as previously. Remarkably, all but one of the 23 clones behaved identically as during its initial growth (III).

The respiration of the G_{TS} clones was also measured at different time points (III, see Fig 5, also V). These results rule out the possibility that the final heteroplasmy levels reached were the result of systematic selection for respiratory competence.

In addition, I tested whether short-term DMSO exposure during cell freezing might have been the cause of rapid mitotic segregation, via cytoskeletal alterations. Different kinds of DMSO treatments were found to have no effect on heteroplasmy levels or distribution. The implications of these results for the intracellular organization of mtDNA is discussed extensively in chapter 6.

5.7 A gain of chromosome 9 associated with heteroplasmic instability (V)

The clone-specific and reproducible shift in genotype was found to be associated with a gain of chromosome 9 (III, V), which was inferred to have taken place at an early time in the derivation of line G_{TS}. The gain of the whole (or most of) chromosome 9 was first detected by comparative genomic hybridization (CGH) and further verified by centromere-fluorescent *in situ* hybridization (FISH) to be a change from disomy to trisomy 9. However, based on a series of co-culture experiments, the gain of chromosome 9 itself did not appear to give a selective growth advantage, at least in cells that had reached stable heteroplasmy values. Furthermore, these experiments indicated that selection due to respiratory phenotype was also minimal.

5.8 Nucleoid organization in heteroplasmic cybrids (V)

Studies of mitochondrial nucleoid organization were carried out using a novel reporter gene construct targeted to nucleoids (Spelbrink et al 2001). The cDNA sequence for the full-length Twinkle protein was fused to EGFP, and then expressed in different cell-lines. This showed a punctuate fluorescence which, as shown by Mitotracker® counterstaining and confocal microscopy, is clearly localized within mitochondria (Spelbrink et al 2001). These structures also contain mtDNA since they can also be stained by ethidium bromide (Spelbrink et al 2001). They were therefore assigned as mitochondrial nucleoids. Expression of this reporter showed no differences in the number or subcellular distribution of nucleoids between suppressor cell-lines containing two or three copies of chromosome 9, and which had exhibited, respectively, heteroplasmic stability and transient mitotic segregation. In both cases, approximately 50-100 nucleoids were found (V).

5.9 Summary of the main experimental findings

The following main conclusions arise from these results:

(1) The G12300A suppressor mutation shows that mtDNA haplotype can have a profound influence on the expression of a disease mutation, even to the level of abolishing its effects completely in one of the cell-lines studied.
(2) The A3243G mutation impairs cell respiration by causing a deficit of translational capacity for Leu(UUR) codons in A549 cells. There may be other effects in other nuclear backgrounds.
(3) The A3243G mutation shows clear threshold effects at the cell level (though at higher amounts of heteroplasmy than seen *in vivo*) which imply that the rate and direction of heteroplasmic segregation could have drastic effects on the phenotypic outcome in the whole body.
(4) At least in cell culture, heteroplasmic segregation is extremely slow (apparent segregation number >1000), yet the number of mitochondrial nucleoids is low, around 50 per cell. This implies that the segregating unit (i.e. the nucleoid) must itself be heteroplasmic, and suggests that resolution of heteroplasmy is almost certainly constrained in some way (e.g. via faithful duplication of nucleoids, as discussed in the next chapter).
(5) This heteroplasmic stability can be (at least transiently) abolished by a change in nuclear karyotype, i.e. an altered pattern of nuclear gene expression. A locus in chromosome 9 is a candidate for involvement in this process.

6. Discussion

Detailed discussions can be found in articles I-V. The major points arising are summarized here. In addition, unpublished results and some new ideas are further discussed below.

6.1 Defects caused by the A3243G mutation (I, II)

Two crucial questions arise concerning the molecular effects of the mutation. Firstly, does it affect mitochondrial protein synthesis, some other process, or both? Secondly, assuming an effect on mitochondrial protein synthesis, is the primary effect a loss of translational capacity or impaired accuracy of translation? In comparing the results that I obtained with those of others, it is clear that differences are seen between cell lines and even in the same cell background, using different techniques.

There appear to be several ways that the A3243G mutation impairs protein synthesis. The results presented in article I demonstrate that, in the A549 cell background, the final cause is the inability to read UUR codons, since protein synthesis can be restored with the suppressor mutation capable of reading the UUR codons. However, this is not the complete story. We know that there is less tRNA-Leu(UUR) in the cells containing the A3243G mutation. There can be several causes for this. The level of transcription may be decreased, or its balance altered. This could be caused by defects in the binding of mTERF or other factors affecting transcriptional activity (Hess et al 1991, though contradicted by Chomyn et al 1992, 2000). RNA processing may also be defective. Indeed, this seems to be the case in the lung carcinoma cybrids, as reported elsewhere for osteosarcoma cell cybrids containing the same mutation (King et al 1992, Schon et al 1992, Koga et al 1995). Normally we see hardly any of the so-called RNA 19, the putative precursor RNA spanning tRNA-Leu(UUR) and containing both 16S rRNA and ND1 mRNA sequences. This applies both to wild-type cells and to cells containing very high levels (>95%) of A3243G mutant mtDNA. However, in the suppressor line there is a significant increase in the amount of RNA 19. It may be surmised that in cells with a high percentage of mutant mtDNA there is a significantly decreased amount of mitochondrial RNA synthesis due to the lack of energy in the form of ATP, but this is not the case in the suppressor line. Protein synthesis, respiration and energy production are effectively normal in the suppressor line, and it is likely that this applies also to RNA synthesis, accounting for the accumulation of

RNA 19 in G_T cells. In addition, it has been shown that the half-life of the mutant tRNA is greatly decreased (Yasukawa et al 2000a).

It is possible that the leucyl-tRNA synthetase does not efficiently recognize the mutated tRNA. In article II it is shown that the aminoacylation level of the mutated tRNA is much lower than that of the wild-type tRNA-Leu(UUR) in control cells. Other laboratories have demonstrated this also (Janssen et al 1999, Chomyn et al 2000). From other studies we know that there are defects also in base-modification of the mutant tRNA (Helm et al 1999, Yasukawa et al 2000b). Mitochondrial tRNAs have several different kinds of base modification, of which the modification of the wobble base (residue 34 in the conventional numbering scheme) explains the relaxed rules for reading the codons of mitochondrial mRNAs. In wild type tRNA-Leu(UUR) the U at position 34 is hypermodified (Sprinzl et al 1998, Yasukawa et al 2000b). This modification restricts the codon recognition to only UUR codons (that is, UUA or UUG). If the modification of U is missing, as on many other mitochondrial tRNAs, the wobble U can apparently decode all four codons of a group. The failure of wobble-base U hypermodification in the mutant tRNA-leu(UUR) can potentially lead to mis-recognition of UUY (that is UUU or UUC) codons that in mitochondria are used to encode phenylalanine. This makes the misincorporation of leucine instead of phenylalanine possible. Indeed, there is at least one patient having the MELAS syndrome apparently caused by a point mutation in the gene for cytochrome *c* oxidase subunit III, where a specific phenylalanine is mutated to leucine (Mandfredi et al 1995). It has also been shown that cell lines containing the A3243G mutation have an altered amino acid incorporation in mitochondria (Flier et al 1997). It would be interesting to test if misincorporation really takes place in human cybrid cells, i.e. by protein sequencing or by raising (peptide) antibodies to different, conserved regions of respiratory proteins containing leucine encoded by UUR codons.

Combining the above findings, it may be proposed that cells carrying moderate levels of the mutation are “saved” from the effects of codon mis-reading by the defects in pre-tRNA processing, stability and aminoacylation that are also caused by the mutation. The fact that only a small amount of aminoacylated mutant tRNA is present would mean that phenylalanine codons would only rarely be mis-read as leucine. Such mis-reading would probably be more deleterious to cells than ribosome stalling during protein synthesis because of the lack of one charged tRNA, at least in cell culture. The charged, mutant tRNA-Leu would have to compete with the significantly greater amount of charged tRNA-Phe. Also it is possible that problems in protein synthesis are a signal for the cell to produce more ribosomes, leading to greater synthesis of the rRNA precursor transcript and increased amount of tRNA-Phe simultaneously, tRNA-Phe being within the rRNA transcription unit of the H-strand.

Variation in the aminoacylation of the mutant tRNA, or the overall ratio between charged tRNAs-Phe and -Leu could potentially explain the wide variety of symptoms caused by mutations in tRNA-Leu(UUR). There could be differences in handling of the mutated tRNA in different nuclear backgrounds, thus resulting in differences both in reading UUR codons and especially in the extent of misincorporation of leucine instead of phenylalanine.

One other possible explanation for the wide variety of patient phenotypes could lie in differences in the amounts of different isoforms of tRNA-Leu(CUN) (II). The levels of the different isoforms appear to be different in different cell backgrounds. If one modified version of tRNA-Leu(CUN) could read also UUR codons, protein synthesis could be close to normal, even in the presence of high levels of the A3243G mutation.

Proteins in mitochondria can apparently move relatively freely inside cells (Nunnari et al 1997, Newman et al 1996, Moraes et al 1999). In a cell containing high levels of mutated mtDNA the efficiency with which abnormal proteins are turned over could also play an important role in the phenotypic outcome. If protein degradation were not working efficiently, polypeptides containing misincorporated amino acids could accumulate and harm the cell more widely than if present in only in one mitochondrion. On the other hand, if protein degradation is efficient, higher amounts of mutated mtDNA molecules may be tolerated (see review by Kaser and Langer 2000).

Abnormal polypeptides can arise by additional mechanisms than misincorporation of aminoacids. Ribosome stalling due to the lack of aminoacylated tRNA-Leu may cause translational frameshifts. This could account for the abnormal protein products made in line G which are, as yet, unidentified. For example, one possibility is that one of the abnormal polypeptides seen in Figure 5.4 and other gels (not shown) could be a fused version of A8/A6. A -1 frameshift at the UUR codon in the overlap region between the A8 and A6 genes would shift translation into the A6 reading frame, missing out the A8 stop codon and creating a fusion protein. The result would then be a nonfunctional polypeptide, with an upstream portion from A8 and a downstream portion from A6, but lacking some amino acids of each. A further frameshift might occur downstream, creating a variety of A8/A6 fusion polypeptides of different sizes. Hardly any A8 is made in these cells, and in addition based on 2-D gel electrophoresis, the assembly of complex V is severely impaired (I). Although the precise function of A8 is unclear, it is clearly required for proper assembly of Complex V (Fearnley and Walker 1986).

Taken together, the findings in this and other studies make it likely that the precise nature and severity of symptoms in patients with A3243G mtDNA are a combination of several different abnormalities in mitochondrial translation, each subject to influences from nuclear factors that could be specific for tissues or individuals.

6.2 Phenotypic suppression by G12300A (I, II)

The data presented in article I, together with the unpublished serial cybridization data shown in this thesis, demonstrate that the point mutation G12300A in the anticodon of tRNA-Leu(CUN) can restore the cellular defects caused by the A3243G mutation. Cell oxygen consumption, mitochondrial protein synthesis and assembly are as normal in G_T cells as in control cells having no trace of either mutation. This restoration is clearest in the lung carcinoma cell background. In the human osteocarcoma cell background there was only a partial restoration of respiratory phenotype, especially in the cells with the highest level of G12300A mutant mtDNA. However, the osteosarcoma cells even with 100% A3243G mutated mtDNA do not show any obvious protein synthesis phenotype, so the issue of quantitative restoration of protein synthesis cannot be evaluated.

The suppressor tRNA cannot restore the respiratory phenotype completely in one of the two nuclear backgrounds tested. Therefore, misincorporation at UUY codons or some other functionally dominant interference with protein synthesis could still be significant as a pathogenic mechanism for the A3243G mutation. The differences in mutant and suppression phenotype between the two backgrounds might be due to differences in the ratio of tRNAs-Phe and -Leu (see above), differences in the representation of the various isoforms of tRNA-Leu(CUN) synthesized in these cells, or in some other difference in nuclear gene expression, affecting, for example, mitochondrial protein degradation. Such differences could reflect underlying genetic differences between individuals or could be linked to development and cell-type. Alternatively they may be trivial consequences of the fact that these are highly abnormal tumour cells that have, moreover, been treated with ethidium bromide, a mutagen, to make them rho^0, hence able to incorporate foreign mtDNA as cybrids.

This kind suppression has never been found *in vivo* in patients. Neither the original patient, nor any other patient nor their healthy family member tested contained detectable amounts of the suppressor mutation. Thus it is likely that mildly affected patients have some other, “natural” suppression mechanism discussed above. However, these studies of the suppressor mutation represent a paradigm for how mtDNA haplotype may modulate, albeit in a less drastic form, the expression of disease mutations.

On the other hand, it is not certain that the suppressor mutation would restore the disease phenotype caused by the A3243G mutation *in vivo*. In the case of the suppressor mutation there is the reciprocal misincorporation problem to that proposed in the case of A3243G-mutated tRNA-Leu(UUR). Naturally the tRNA-Leu(CUN) can read all the four

possible codons, that are CUA, CUG, CUU and CUC. In the case of unmodified but G12300A-mutated tRNA-Leu(CUN), it is thus possible that it would recognize UUY phenylalanine codons and insert leucine instead of phenylalanine, as illustrated in Figure 6.1.

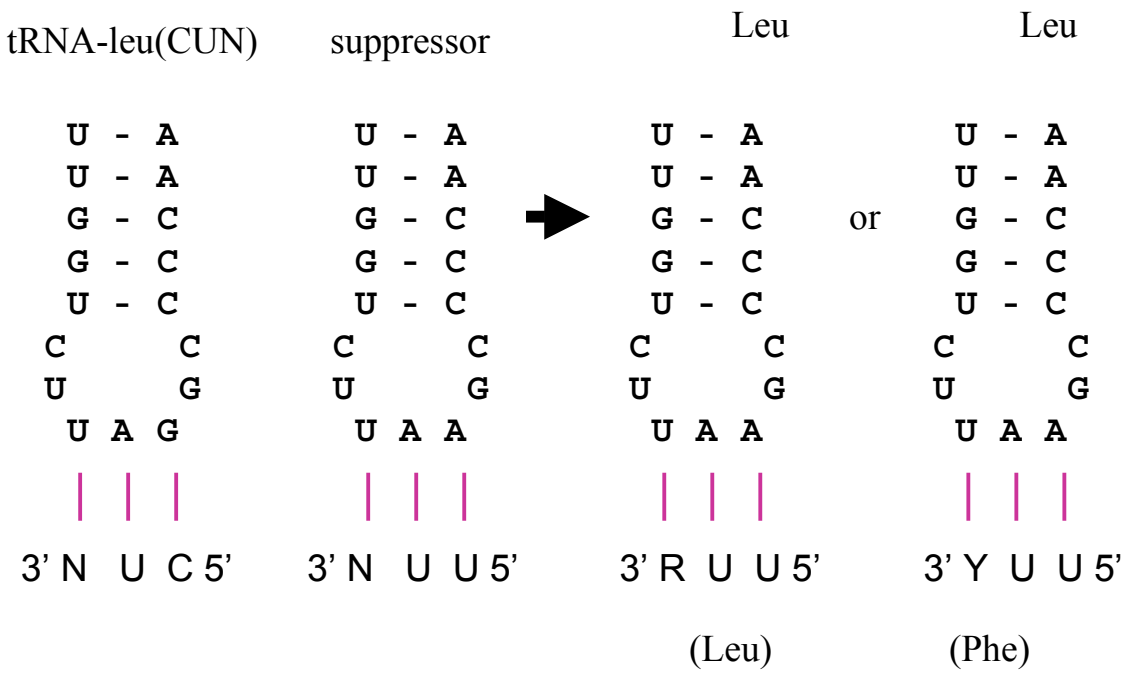


Figure 6.1. Possible misreading of UUY codons by the unmodified suppressor tRNA. Only the anticodon stem of each tRNA is shown.

Even though cells can tolerate a wide range of levels of the suppressor mutation, problems arise when the level of the mutation reaches near 70% or more, even in the lung carcinoma background. This is clearly a lower threshold than is the case for pathological mitochondrial tRNA mutations commonly seen in cybrid cell models. The A3242G mutation itself shows a threshold of around 95% before manifesting a clear phenotype. This would support the view that a low-level amino acid misincorporation does take place, although it is possible that the threshold effect is due to reduced capability of recognition of CUN codons, since for some tRNAs, mutations that merely reduce the tRNA level to 20-30% of control values do have detectable effects on cells in culture (Guan et al 1998, Toompuu et al 1999). Interestingly, cells adapted to the suppressor mutation apparently cannot survive the loss of the mutation, unless there is simultaneously an increase in mtDNA wild-type at np 3243 to at least 5-10%, or at least these cells are unclonable.

6.3 The mitochondrial unit of inheritance (III, IV, V)

The results presented in this thesis and its accompanying articles support the emerging view of a multi-copy mitochondrial nucleoid as the fundamental unit of organization and inheritance of mtDNA. This involves a re-interpretation, rather than a rejection of previous studies, where the unit of mitochondrial inheritance was assumed to be a single mitochondrion (Attardi et al 1995), or even a cell. Article III shows that the segregation number is high. Article V, taken together with Spelbrink et al 2001, shows that the number of nucleoids is much lower, by a factor of at least 10 and possibly 100. The model reconciles these observations.

The "faithfully duplicated nucleoid" model (article IV) can explain the phenomena seen in article III. The model proposes that the nucleoid consists of a permanent set of mtDNA molecules, that can be different from each other (heteroplasmy), and are faithfully replicated as a unit, via a process akin to mitosis (see Figure 2 in IV). The heteroplasmy level of daughter nucleoids remains the same from division to division. In a stably heteroplasmic cell the heteroplasmy level is proposed to be approximately the same in all nucleoids, even if not every single nucleoid is replicated in a given cell division round. Most importantly, faithful duplication of nucleoids ensures that somatic mutations remain masked by intranucleoid complementation. However, the maintenance and faithful replication of nucleoids is still only a hypothesis. So far it is also not understood how nucleoid organization might be kept invariant.

Under normal circumstances, the mitochondrial genome is stable over long periods in non-selective cell culture (III). Even cells having a high mutation load do not shift in their genotype. The segregation number of >1000 is strikingly greater than earlier studies in the mouse germ line, where the number of segregating units was shown to be approximately 200 (Jenuth et al 1996). It is likely that, in the germ-line, there is a transient reduction in copy number, accompanied perhaps by reorganization of nucleoids, such that only one or just a few nucleoids are replicated and segregated into dividing cells (Shoubridge 2000). This explains the so-called bottle-neck phenomenon and could account for the homoplasmic status of the mitochondrial genome normally seen in humans. However, disease-causing mutations are most often seen in the heteroplasmic state. There seems to be little oxidative selection in embryos, at least for the heteroplasmy levels we can observe (see review by Shoubridge 2000). Of course it is possible that selection works only at extreme heteroplasmy levels, therefore no homoplasmic individuals are ever seen.

It is possible that, over the course of evolution, it has been beneficial to be able to keep mitochondrial nucleoid organization constant during development. In very early steps

of development the nonfunctional mitohondria (or cells or nucleoids) are selected out and only functional mitochondria (or cells or nucleoids) remain to survive and be replicated. On the other hand, if constant reorganization occurs, it is possible to end up having nucleoids full of mutations leading to mitochondrial dysfunction and probably apoptosis later in development. This might be deleterious to the organism.

Radical nuclear alterations can occur occasionally, especially in cancer cells. It may be by chance that mitochondrial DNA can reorganize simultaneously with the alterations in nuclear genes. For instance, cytoskeletal aberrations could cause chromosome non-disjunction and abnormal mtDNA partition at the same time. Hypothetically there might also be a programmed mechanism for a cancer cell to reorganize mitochondrial nucleoids and then select the new kind of mtDNA for altered cells. Signals inducing this behaviour could be from altered nuclear gene expression, that results from deletions or duplications of nuclear chromosomes, or even coming as external signals from neighbouring cells. Because a cancer cell divides aggressively we may eventually detect the new kind of mtDNA in a cancer tissue (Bianchi et al 2001). A novel L-strand transcript from the D-loop has been found in cancer cells (Duncan et al 2000) indicating that indeed, in cancer cells, some unidentified processes may take place.

Of course, it is possible, that the results shown in papers III and V (either/both the stable heteroplasmy with high segregation number or/and transient shift in heteroplasmy level followed by re-establishment of stability) are just an example of processes that can be seen in abnormal cancer (lung carcinoma) cells. Alternatively, it is possible that faithful nucleoid replication is a response to the presence of pathological mutations. Therefore, I think it would be important to repeat these experiments using neutral mtDNA polymorphisms.

6.4 Transient instability of heteroplasmy (III, IV, V)

It is proposed that, under some circumstances, uniform, heteroplasmic nucleoids can break down, followed by reorganization or *de novo* reformation of nucleoids. During this kind of an event, mtDNA is propagated and partitioned by some other unknown mechanism. Uniform, heteroplasmic nucleoids would be replaced by different kinds of nucleoids containing variation in heteroplasmy levels within a single cell. Such a sequence of events is proposed to have occurred in the G_{TS} cell line which became trisomic for chromosome 9. After the suggested nucleoid breakdown and re-formation, only some nucleoids in each cell may have replicated, or mitotic segregation may simply have

purified the genotype of the cell in a relatively small number of cell generations, the number of segregating units during this process being equal to the number of nucleoids. Either mechanism would restore uniform nucleoid composition in the cell after enough cell divisions. During this proceses, selection can also operate, and harmful mutations are more likely to disappear. Rarely, the opposite might happen (Kobayashi et al 1992, Yoneda 1992, Holt et al 1997, Vergani et al 1999), or a mutation beneficial at low levels may accumulate to a higher level at which it is harmful. This appears to have occurred for the suppressor mutation in some G_{TS} clones where it segregated to levels of 70% or more, thus impairing respiration.

This kind of shift to an apparently 'worse' genotype can be explained in several different ways. The first is equivalent to the so-called "crippled mitochondrion" hypothesis (Yoneda et al 1994), which suggests that energetic problems are a signal for the nucleus to invest more in mitochondria. There may also be factors favouring the replication of certain types of DNA molecules, although their identity is unknown. Of course, this can be a stochastic phenomenon: no selection either way, but just some clones in which random segregation drives the heteroplasmy level up, and in others down. However, in the last case it would be surprising to see heteroplasmy levels going up and down in a reproducible fashion, as was seen for individual cell clones.

6.5. Differences between cell-types (III, IV, V)

6.5.1 Different cell lines

Where a shift in mtDNA heteroplasmy takes place, its direction appears to be nuclear background dependent (Dunbar et al 1995). This direction of shift in genotype could be due to different nucleoid organization, since it might be possible that nucleoids containing less DNA are more sensitive to mutations. Therefore, some kinds of nucleoids would be lost due to selection. The number of nucleoids appears to be different between lung carcinoma and osteosarcoma cells, based on reporter assays (Spelbrink et al 2001, V).

Nucleoid organization should have more widespread effects too. We know, that cells having a long life-span have more mtDNA that short-lived cells, such as sperm or leukocytes. It is possible that there is more DNA in a single nucleoid as well. If a nucleoid contains only a few mtDNA molecules, accumulation of just a few deleterious mutations could compromise the genetic fitness of the whole nucleoid. If unfit nucleoids became prevalent the cell would be likely to undergo cell death. On the other hand, if there were

many molecules in a single nucleoid, the deleterious effect of a single mutation can be masked by complementation. So the large amount of DNA molecules is a safety mechanism in mitochondria.

6.5.2. Changes in nuclear karyotype

It is also possible that a massive change in nuclear karyotype *per se* would be a cause of relaxed mtDNA partition rules. A typical feature of a cancer cell is uncontrolled cell division, and such cells are under continuous selection for improved growth. Therefore, massive nuclear changes may cause some kind of cellular crisis that automatically signals an SOS response in the cell. A transient reorganization of the mitochondrial genome may be an inbuilt part of such a response, essential to permit new combinations of nuclear and mitochondrial genotype to arise in favour of cell survival. Of course, this may be more a consequence than a cause, since cytoskeletal organization is almost certainly essential for both mitochondrial genome stability and chromosome movement.

6.5.3. Pitfalls of cybrid technology

Understanding the meaning of nuclear background associated with mitochondrial mutations is essential for understanding the pathology of mitochondrial disease and mitochondrial function more generally. However, there are not many possibilities for controlled studies. Thus far, cybrid technology, i.e. the fusion of rho^0 cells (cells without mitochondrial DNA) with the enucleated cytoplasm derived from the cells of a patient, has been the only way to study the effect of a pathological mtDNA mutation in different cell backgrounds, or to study different mutations in the same nuclear background. In addition, this technique has enabled us to study segregation and complementation of mtDNA. However, the study presented here shows how careful we should be in drawing conclusions when using cybrid cells. Such studies are unavoidably based on tumour-derived cell-lines. These cells undergo a huge number of cell divisions, and lose accurate control of DNA maintenance in the nucleus. That results in gross nuclear DNA rearrangements, polyploidy, deletions, duplications and mutations. Therefore, in my opinion, we should not so frequently use phrases like ”controlled nuclear background”, or “same nuclear background” or not even “same parental cell-line”, since too many differences accumulate during culture and also between laboratories all around the world.

There is also a significant danger of drawing erroneous conclusions. What if the suppressor phenotype (line G_T) had been found only after the gain of chromosome 9 (line G_{TS}). Maybe the finding of trisomy 9 would had led us towards the idea that changes in chromosome 9 gene dosage can suppress the defect caused by the A3243G mutation. We might still be following that futile hypothesis instead of thinking about a possible effect of trisomy 9 on segregation and nucleoid organization. And of course, the hypothesis that trisomy 9 is directly linked to transient segregation may itself be proved false.

These studies show that cybrid cells can, with appropriate controls, be used for very short-term experiments. Replicate experiments should preferably be set up simultaneously, or alternatively, using cells frozen earlier at a defined passage. Freezing cells in all the steps is crucial. Also, in my opinion, continuous monitoring of both mitochondrial genotype and nuclear karyotype is important. For any longer-term experiments, the interpretation of results should be done very cautiously.

On the other hand, it is undeniable that the use of cybrid technology has resulted in several important findings reported here, as elsewhere. Firstly, the appearance and characterization of the G12300A suppressor mutation has permitted a much deeper understanding of the pathological mechanism of the A3243G mutation. It has also indicated the potential importance of mtDNA haplotype in the expression of mitochondrial disease mutations, and has given insights into more general issues in mitochonodrial translation. Secondly, the gain of chromosone 9 in cell culture, associated with transient heteroplasmic segregation, has led to a new hypothesis concerning the organization and maintenance of mitochondrial DNA. These insights have come about as a result of following up unexpected and initially even counter-intuitive findings. One wonders how many other such findings have been “brushed under the carpet” in the course of cybrid studies.

7. Conclusions

The study presented here was aimed at elucidating the pathological effects of the A324G mutation at the molecular level, at investigating possible mechanisms for its phenotypic suppression and at understanding the maintenance and segregation of mtDNA heteroplasmy.

My main conclusions are:

1) In the lung carcinoma cell background, the A3243G mutation decreases protein synthesis inside mitochondria, leading to the synthesis of abnormal polypeptides and defects in the assembly of the respiratory chain complexes. Abnormal RNA processing, reduced amounts of tRNA-leu(UUR), and reduced aminoacylation of the tRNA are features of the mutant phenotype in this background. The protein synthesis defects can be corrected by heteroplasmy for the G12300A mutation, which creates a suppressor tRNA capable of reading UUR codons inside mitochondria. This indicates that the major molecular effect of the A3243G mutation in A549 cells is a deficiency of decoding capacity for UUR codons.

2) Nevertheless, the extent of suppression by the G12300A mutation appears to depend on nuclear background, suggesting that in some circumstances, the A3243G mutation might have additional, deleterious effects on protein synthesis. One possibility would be via misreading of UUY (Phe) codons by the mutated tRNA-Leu(UUR). The suppressor tRNA could itself have some deleterious effects. The decreased level in the total amount of aminoacylated tRNA-Leu(UUR) might, conversely, be a safety mechanism to prevent possible misreading. In addition, the relative amounts of differently modified forms of tRNA-Leu(CUN) may provide a natural suppression mechanism in A3243G mutated cells, which could also be nuclear background dependent.

3) The segregation number for stably heteroplasmic lung carcinoma cells is >1000, at least an order of magnitude greater than in the mouse germ-line, and also much greater than the number of mitochondrial nucleoids in these cells. If the unit of mitochondrial inheritance is the nucleoid, it follows that the nucleoid must itself be heteroplasmic in such cells.

Furthermore, long-term stability of heteroplasmy at the cell level implies that heteroplasmic nucleoids must be faithfully duplicated, to prevent mitotic segregation.

4) Heteroplasmic cells can nevertheless undergo episodes of transient heteroplasmic instability, re-stabilizing at new heteroplasmy levels. The outcome appears not to be the result of systematic selection based on respiratory phenotype, rather it reflects some stochastic process. It is suggested to involve a reorganization of heteroplasmic nucleoids, such that they acquire diverse levels of heteroplasmy. Mitotic segregation then re-purifies the nucleoid population of each cell.

5) A shift from disomy 9 to trisomy 9 in cultured lung carcinoma cybrid cells was found to be associated with one such episode. This suggests that a locus on chromosome 9 is associated with maintaining the internal organization or duplication of nucleoids or with maintaining a stable mitochondrial genotype by some other mechanism.

7) Although cybrid technology is a very powerful tool for analysing the defects caused by mitochondrial mutations, a great deal of caution must be exercised in drawing conclusions. Tumour-derived cells are aneuploid, and their karyotypes can change during growth in culture, with effects on mtDNA organization, mitotic segregation or even on the phenotypic expression of the mutant phenotype. Constant monitoring of both nuclear and mitochondrial genotypes is advisable, even though it is laborious.

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