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Human disorder in energy transduction – molecular pathology

THESIS ADDENDUM

Page xi, (Publications).
Correction: publication 3 should read accepted in Hum. Genet., not accepted in Am. J. Hum. Genet.

Page xii. Add as a new publication:
"5. **Malik S.**, Sudoyo H., Pramoonjago P., Sukarna T., Darwis D., Marzuki S. Evidence for the *De Novo* Regeneration of the Pattern of the Length Heteroplasmy Associated with the T16189C Variant in the Control (D-Loop) Region of the Mitochondrial DNA. J. Hum. Genet. 2002, 47: In press."

Page 5, paragraph 2. Add at the end of line 6:
"The involvement of an environmental factor(s) are best illustrated by two examples: (a) in Leber's hereditary optic neuropathy (LHON) associated with mtDNA G11778A mutation, and (b) in the sensorineural hearing loss associated with the mtDNA A1555G mutation. Cigarette smoking has been suggested to be a precipitating factor in LHON; e.g. a significant association was observed between smoking and disease penetrance in a LHON pedigree (Tsao et al. 1999). And the phenotypic expression of the causal mtDNA mutation in the case of sensorineural hearing loss associated with mtDNA A1555G mutation is precipitated by exposure to aminoglycoside antibiotics (Prezant et al. 1993) as further elaborated in Chapter 3. The involvement of a nuclear factor(s) is indicated by the incomplete penetrance and male predominance observed in pedigrees with LHON (see Chalmers and Schapira 1999), as well as the low penetrance of the causal mtDNA mutation in families with non-aminoglycosides induced deafness (Prezant et al. 1993; Matthijs et al. 1996). The issue of nuclear-mitochondria interplay is the main theme of this thesis, in particular the study of the mtDNA length polymorphism described in Chapter 4."

Page 40. Add at the end of paragraph 1:
"Thus, pathological mtDNA mutations basically lower the biochemical threshold to a level, resulting in a much earlier expression of (perhaps accelerated) age-related accumulation of mtDNA mutations."

Page 158. Add at the end of paragraph 2:
"The low level of this nine poly [C] morph that still can be observed is probably due to the remnants of the mtDNA species or of the DNA replication machinery (i.e. the DNA polymerase γ) from the mitochondrial donor still remaining at the early stage of the cybrids growth; further growth of the cybrids would deplete the cells of this particular morph."

Page 182. Add a new reference at the end of the page:
"Chalmers RM, Schapira AH (1999) Clinical, biochemical and molecular genetic features of Leber's hereditary optic neuropathy. Biochim. Biophys. Acta 1410:147-158."

Page 218: Add a new reference after Trounce et al (1994):
"Tsao K, Aitken PA, Johns DR (1999) Smoking as an aetiological factor in a pedigree with Leber's hereditary optic neuropathy. British J. Ophthal. 83:577-581."

HUMAN DISORDER OF ENERGY TRANSDUCTION – MOLECULAR PATHOLOGY

A thesis presented for the degree of

Doctor of Philosophy

by

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July, 2001

To Sarsono, Sasha and Satyo

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DECLARATION

This thesis contain no material which has been accepted for the award of any other degree or diploma in any university; and, to the best of my knowledge, contains no material previously published or written by another person, except where due reference is made in the text.

[redacted]

Safarina Golfiani Malik

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PUBLICATIONS

Some of the work described in this thesis have been communicated in the following publications and abstracts:

Publications:

1. Sudoyo H, Sitepu M, **Malik S**, Poesponegoro HD, Marzuki S. Leber's hereditary optic neuropathy in Indonesia: two families with the mtDNA 11778G>A and 14484T>C mutations. Hum Mutat. 1998. Suppl 1:S271-4.

2. **Malik S.**, Sudoyo H. and Marzuki S. Microphotometric Analysis of Fibroblast from Leber's Hereditary Optic Neuropathy Patients with 11778 G→A Mitochondrial DNA Mutation. J. Inherit. Metab. Dis. 2000. 23:730-744.

3. **Malik S.**, Sudoyo H., Pramoonjago P., Suryadi H., Sukarna T., Njunting M., Sahiratmadja E., Marzuki S. Nuclear Mitochondrial Interplay in the Modulation of the Homopolymeric Tract Length Heteroplasmy in the Control (D-loop) Region of the Mitochondrial DNA. Am. J. Hum. Genet. Accepted for publication.

4. **Malik S**, Sudoyo H., Sasmono T., Arhya I.N., Pramoonjago P., Winata S. and Marzuki S. Congenital Non-Syndromic Sensorineural Deafness In A Balinese Family Associated With A1555G Mutation In The

Mitochondrial Small Subunit Ribosomal RNA. Submitted for publication.

Communications to learned societies:

1. **Malik S**, Vaillant F., Marzuki S. and Lawen A. 1997. Interrelationship between Plasma Membrane NADH-Oxidoreductase and Mitochondrial DNA. 22nd Conference on Protein Structure and Function. Lorne. Australia.

2. **Malik S**, Sasmono T, Arhya I.N., Winata S, Pramoonjago P, Sudoyo H. and Marzuki S. 1997. Molecular and Biochemical Defects Underlying the Congenital Deafness In A Balinese Family. IMCB 10th Anniversary Symposium. Singapore. P41.

3. **Malik S.**, Sudoyo H., Marzuki S., Lawen A. 1997. Is There Any Relationship between Mitochondrial Electron Transport Chain and Plasma Membrane NADH-Oxidoreductase? 8th Indon. Biochem. Mol. Biol. Conference. Surabaya. Indonesia. C28

4. Sudoyo H., Pramoonjago P., **Malik S.**, Suryadi H., Sukarna T. and Marzuki S. 1999. Characteristics of the Length Heteroplasmy Associated with a Disease-Related Sequence Variant in the Control (D-

Loop) Region of the Mitochondrial DNA. 2nd Asia-Pacific IMBN Conference. Singapore. P30.

ABBREVIATIONS

adPEO	autosomal dominant progressive external opthalmoplegia
ANT1	adenine nucleotide translocator 1
BSA	bovine serum albumin
CPEO	chronic preogressive external ophthalmoplegia
D-loop	displacement loop
EDTA	ethylenedieamine tetraacetic acid
EGTA	ethylene glycol bis (beta-aminoetyl eter) NNNN tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
Glut.	glutaraldehyde
HEPES	N-2-hydroxy ethylpiperazine-N'-2-
HVR	Hypervariable region
KLH	Keyhole limpet hemocyanin
LHON	Leber's hereditary optic neuropathy
MDM	Mitochondrial diabetes melitus
MELAS	Mitochondrial encephalopathy lactic acidosis and stroke-like episodes
MERRF	Myoclonic epilepsy with ragged red fibers
mtDNA	mitochondrial DNA
NADH	nicotinamide-adenine dinucleotide (reduced)
NADH-CoQ	NADH coenzyme-Q oxidoreductase
NADH-TR	NADH-tetrazolium
ND	NADH dehydrogenase
PAB	para-amino benzamidin
PMSF	phenyl methyl sulfonil fluoride
ROX	reactive oxygen species
rRNA	ribosomal RNA
SDS	sodium dodecyl sulphate
SEA	South East Asian
SNP	single nucleotide polymorphism

SUMMARY

This thesis describes a series of three studies, which are concerned with aspects of the molecular pathology of the human respiratory chain disorders. In summary, the following results have been obtained:

1. **MICROPHOTOMETRIC ANALYSIS OF NADH-TETRAZOLIUM REDUCTASE DEFICIENCY IN FIBROBLASTS OF PATIENTS WITH LEBER'S HEREDITARY OPTIC NEUROPATHY.** A microphotometric procedure has been developed to measure the mitochondrial complex I activity histochemically, and the validity and the linearity of the NADH-tetrazolium reductase activity have been verified. Arrhenius kinetics study for the NADH-tetrazolium reductase shows that the plot exhibits a break at membrane lipid phase transition temperature at 13°C indicative of the membrane association of the enzyme complex. The NADH-tetrazolium reductase in both fibroblast cell lines with 11778 LHON mutation have been measured, and compared the activity to normal fibroblast lines. The activity is reduced by approx. 30% in LHON cell lines. The study presented in this chapter demonstrate the utility of the microphotometric analysis in the study of enzyme deficiency at the single cell level. Such information is important, for example, when mosaicity is expected in the expression of a pathological condition, such as that resulting from a mutation in the mtDNA.

2. CONGENITAL NON-SYNDROMIC SENSORINEURAL DEAFNESS IN A BALINESE FAMILY ASSOCIATED WITH MUTATIONS IN THE MITOCHONDRIAL SMALL SUBUNIT RIBOSOMAL RNA. MtDNA associated disorders behave more like polygenic diseases, in that factors encoded in the mitochondrial and nuclear DNA, as well as environmental factor, play a role in modulating the expression of the causal mtDNA mutations. This Chapter reports a large family of Balinese origin of Southeast Asian (SEA) background carrying the mtDNA A1555G mutation expressed as familial progressive/congenital sensorineural deafness. The family exhibited a complex inheritance pattern shown to be the result of an unusual multiple entry of the mutation into the pedigree, perhaps associated with the social-cultural setting of the Balinese village. Despite the phylogenetic distance of the SEA mtDNA, which provide a background for the A1555G mutation in the Balinese family, the genetic characteristics and biochemical expression of the A1555G mutation is basically similar to those in the Caucasian and Japanese. Examination of the respiratory enzyme activities revealed a significant decrease in the respiratory complex I activity in particular in the symptomatic members. Attempt was made to get some insight into the factor(s) that modulate the expression. The complete sequence of Balinese individuals is reported. These are the first SEA mtDNA reported and showed seven novel single nucleotide polymorphism sites (SNPs).

3. NUCLEAR MITOCHONDRIAL INTERPLAY IN THE MODULATION OF THE HOMOPOLYMERIC TRACT LENGTH HETEROPLASMY IN THE CONTROL (D-LOOP) REGION OF THE MITOCHONDRIAL DNA. The genetic characteristics of a homopolymeric tract length heteroplasmy associated with the 16189C variant in the mtDNA control region were studied to identify the factor(s) involved in the generation of the length heteroplasmy. The relative proportion of the various lengths of polycytosines (i.e. the pattern of the length heteroplasmy) is maintained in an individual, and evidence obtained show that it is regenerated *de novo* following cell divisions. The pattern is similar in maternally related individuals, suggestive of mtDNA determinants. Of the 38 individuals with the 16189C variant studied, 39% were found to exhibit the ^{16180}AAACCCCCCCCCCC^{16193} variant associated with A16183C polymorphism ([11C]-group), while 53% showed the ^{16180}AACCCCCCCCCCCC^{16193} variant associated with a further A16182C polymorphism ([12C]-group). Haplotype analysis of the mtDNA revealed the specific association of the longer mean length of the poly [C] in the [12C]-group with haplogroup B. A similar association was also observed in the [11C]-group, but with a novel haplogroup Z. Cybrid constructions revealed that nuclear factor(s) involvement in the generation of the length heteroplasmy is prominent in homopolymeric tract of eight cytosines. The nuclearly coded factor(s) is/are presumably related to the fidelity of the nuclearly coded components of the mitochondrial DNA replication machinery.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTORY REMARKS

Mitochondrial oxidative phosphorylation (OXPHOS) is one of the critical and essential process in living organism because of its importance for the cell viability and survival. The most important function of the mitochondria is the conversion of the chemical energy released during the aerobic oxidation of reduced carbon compounds into ATP through oxidative phosphorylation (OXPHOS), which provides most of the energy that cells require to function. The enzyme complexes responsible for this energy metabolism are located within the mitochondrial inner membrane, and are assembled from polypeptides encoded and synthesized by both the nucleo-cytoplasmic system and a much smaller genetic system located within the mitochondrion. The biogenesis of these enzyme complexes, therefore, involves the coordinate interplay between the two genetic systems. The mammalian mitochondrial DNA (mtDNA) encodes 13 polypeptides, all of which are subunits of the mitochondrial respiratory enzyme complexes. It also encodes the 12S and 16S rRNA and the 22 tRNA required for mitochondrial protein synthesis. The remaining mitochondrial enzyme subunits, the metabolic enzymes, the DNA and RNA polymerases, the ribosomal proteins, and the mtDNA regulatory factors are all encoded by nuclear genes (see Wallace 1999).

The role of mtDNA mutations as the underlying molecular defects in a wide range of human diseases is now well established. It has become apparent in recent years, however, that mtDNA associated disorders behave more like a polygenic disease, in that the expression of the causal mutations are frequently modulated by their nuclear and mitochondrial DNA backgrounds as well as by environmental factors. Enzymatic biochemical disorders consequential of mtDNA mutations and their clinical manifestations appear to be the outcome of an interplay between the causal mutations and other sequence variants of the mtDNA, and between the nuclear and the mitochondrial genetic systems (see Hanna and Nelson 1999; Wallace 1999; Zeviani and Antozzi 1992). Further, the processes involved in the generation of these mutations and their pathobiology are still not well understood. Many questions remain, such as those concern with factors that influence the generation of mtDNA mutations and the pathobiology of diseases as the consequence of the mtDNA mutations.

The series of study described in this thesis were initiated in 1996, with the development of a microphotometric approach to study the deficiency of the respiratory complex I at the single cell level. Respiratory enzyme deficiencies observed in tissues as the consequence of pathological mtDNA mutations, and those observed in ageing tissues, are in general of a mosaic nature. Important information regarding the phenotypic expression of mtDNA mutations, therefore, could be gained from the ability to analyze biochemical and molecular defects at the single cell level. The result of this study, carried out in fibroblast cells carrying the mtDNA G11778A mutation in the ND4 gene, expressed as Leber's hereditary optic neuropathy (LHON), is described in Chapter 2. With the microphotometric approach being well established, I then looked at another disease associated with the mtDNA mutation, in which the interplay of the mitochondria, the nuclear and the environmental factors is more prominent. Around this time, a large Balinese family showing congenital sensorineural deafness associated with mtDNA A1555G mutation was referred to our laboratory. The interesting feature about this family is that it was and still is the only family with the A1555G mutation reported from Southeast Asia; mtDNA of Southeast Asians have been shown to be genetically distant from those of the Caucasian and Japanese, from which most of the cases have been reported. The genetic characteristic of the

A1555G mutation under the influence of the Southeast Asia mtDNA background and its consequential biochemical expression is described in Chapter 3.

The main body of this thesis has been focused on the elucidation of the factor(s) responsible for error generation in mtDNA replication, important to our understanding of the generation of pathological mtDNA mutations and mtDNA normal variants. The significantly higher rate of mutations in the D-loop control region of the mtDNA makes it an ideal region to study the interplay of the factors involved in the generation of mtDNA mutations as a result of error made during mtDNA replication. Of particular interest is a homopolymeric tract associated with a normal variant T16189C in the D-loop region of the mtDNA. The homopolymeric tract comprises ten cytosines that is unstable. Length heteroplasmy of the tract observed, presumably as the result of replication slippage, is modulated by both mtDNA and nuclear factor(s). The T16189C variant has been suggested also to be a predisposing factor to diabetes mellitus, low birth rate and dilated cardiomyopathy. The study conducted to elucidate the nuclear-mitochondrial interplay in the generation of the length heteroplasmy is described in Chapter 4.

To put the present work in perspective and to provide a general background information for the present study, a brief overview of the current literatures is presented. This literature review focuses on the process involved in the biogenesis of the organelle, the communication and coordination between the nuclear and mitochondrial genetic systems, as well as the human diseases associated with mtDNA mutations. Many other important aspects of mitochondrial function and biogenesis, which are of less immediate relevance may not be covered. Excellent reviews, which provide more extensive and detailed discussion are available (Gray 1999; Leslie et al. 1999; Neupert 1997; Taanman 1999; Tokatlidis and Schatz 1999; von Heijne 1999).

OVERVIEW OF THE MITOCHONDRIAL BIOGENESIS

Structure and Function of the Mitochondria

Mitochondria are small, oval shaped organelles surrounded by two highly specialized lipid bilayer membranes, the inner membrane and the outer membrane, with two soluble fractions, the matrix and the intermembrane space. Mitochondria are the sites of aerobic respiration, mainly located in the inner membrane and matrix, and are generally the major energy production center in eukaryotes. Mitochondria generate ATP via OXPHOS, which occurs in the matrix site of the inner mitochondrial membrane. The mitochondrial oxidative phosphorylation system is composed of five enzyme complexes, embedded in the inner membrane (Fig. 1.1). These enzyme complexes are: the NADH-ubiquinone oxidoreductase (complex I), succinate-ubiquinone oxidoreductase (complex II), ubiquinol-cytochrome *c* oxidoreductase (complex III), cytochrome *c* oxidase (complex IV) and the ATP synthase (complex V, H^+-ATPase). Together with two mobile electron carriers, the ubiquinone and cytochrome *c*, complex I, II, III and IV construct the respiratory chain system. At three stages along the respiratory chain, oxidative energy conservation takes place via coupled vectorial proton translocation and the creation of a transmembrane electrochemical potential of protons. The electrons from hydrogens and NADH are transferred through complex I to ubiquinone (CoQ). The electrons from succinate in the Krebs (tricarboxylic acid/TCA) cycle are transferred through complex II also to CoQ. From ubiquinol, the electron are passed to cytochrome *c* by complex III, then finally to oxygen by complex IV. The energy released is used to pump protons (H^+) out of the mitochondrial inner membrane through complexes I, III, and IV to create an electrochemical gradient ($\Delta\psi$). The proton motive force is then utilized to drive the ATP synthase (complex V) to condense ADP and P_i to form ATP. Both ATP and ADP are exchanged by adenine nucleotide translocator (ANT) across the mitochondrial inner membrane (see Fig. 1.1) (see Hatefi 1985).

Subunits of the Respiratory Enzyme Complexes

The properties and subunits composition of the human mitochondrial respiratory complexes are shown in Table 1.1.

NADH-ubiquinone oxidoreductase (complex I). The respiratory complex I is the largest enzyme complex of the mitochondrial respiratory chain system. Subunits structure of this enzyme complex are best documented in *Bos taurus* (Walker 1992), *Neurospora crassa* (Hofhaus et al. 1991), and *Escherichia coli* (Friedrich et al. 1998; Weidner et al. 1993). Bovine heart complex I contains approx. 41 subunits with a

Figure 1.1. Schematic diagram of OXPHOS of the Respiratory Chain System in the Lipid Bilayer Environment.

The mitochondrial oxidative phosphorylation (OXPHOS) system compose of five enzyme complexes are embedded in the lipid bilayer of the inner mitochondrial membrane. These enzyme complexes comprises the NADH-ubiquinone oxidoreductase (complex I), succinate-ubiquinone oxidoreductase (complex II), ubiquinol-cytochrome *c* oxidoreductase (complex III), cytochrome *c* oxidase (complex IV) and the ATP synthase (complex V, H^+-ATPase). Complexes I, II, III and IV construct the respiratory chain system, with the help of two mobile electron carriers, the ubiquinone and cytochrome *c*. The oxidative energy conservation takes place via coupled vectorial proton translocation and the creation of a transmembrane electrochemical potential of protons at three stages along the respiratory chain. Electrons from NADH are passed to complex I, which transferred to ubiquinone (CoQ). Complex II catalyzes electron transfer from succinate in the TCA cycle also to CoQ. Then the electrons are transferred from ubiquinol to cytochrome *c* by complex III, and finally to oxygen by complex IV, releasing energy, which is used to pump protons (H^+) out of the mitochondrial inner membrane through complexes I, III, and IV creating an electrochemical gradient ($\Delta\psi$). The proton motive force is used to drive the ATP synthase (complex V) to condense ADP and P_i to form ATP. Both ATP and ADP are exchanged by adenine nucleotide translocator (ANT) across the mitochondrial inner membrane (adapted from Wallace 1992).

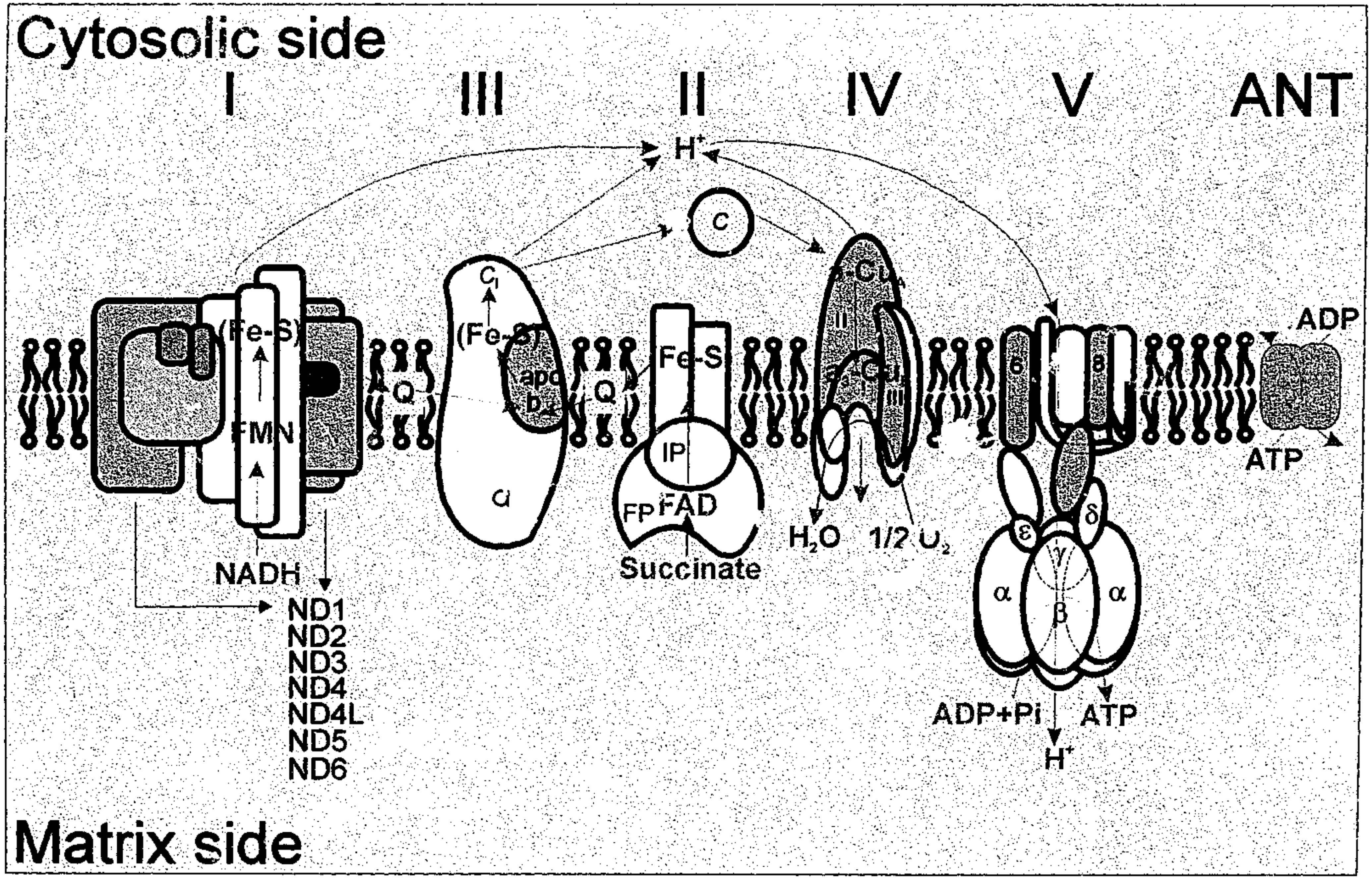

Cytosolic side
I
III
II
IV
V
ANT
H⁺
c
C1
(Fe-S)
apo b
Q
(Fe-S)
FMN
Q
Fe-S
IP
FP
FAD
Succinate
a
a-CuA
II
a3-CuB
III
H₂O
1/2 O₂
6
8
ε
δ
γ
α
β
α
ADP+Pi
ATP
H⁺
ADP
ATP
NADH
ND1
ND2
ND3
ND4
ND4L
ND5
ND6
Matrix side

Table 1.1. Subunits of the Mitochondrial Enzyme Complexes

Complex	Reaction	Subunits		
		Total	Nuclear-encoded	Mitochondria-encoded
I	NADH → CoQ	± 43	± 36	7
II	Succinate → CoQ	4	4	0
III	Reduced CoQ → cytochrome *c*	11	10	1
IV	Ferrocytochrome *c* → oxygen	13	10	3
V	ADP + P_i → ATP	± 16	± 14	2

molecular mass of approx. 10^3 kDa (see Hatefi 1985; Walker et al. 1992). In humans, the exact number of complex I subunits is still unknown. Seven of complex I subunits are mitochondrial-encoded (Chomyn et al. 1986), and at least 36 are nuclear-encoded (34 of these have been characterized) (see Smeitink et al. 1998; Smeitink and van den Heuvel 1999). Mitochondrial complex I has an L-shaped configuration of which the peripheral arm partly protrudes in the mitochondrial matrix (Grigorieff 1998).

Most of the electron carriers, such as flavine mononucleotides (FMNs) and iron-sulfur clusters, are located in the peripheral arm. The complex can be separated into three different fractions when treated with chaotropic agents, the water-soluble flavo-protein (FP) and iron-sulfur protein (IP) fractions, which can be fractionated one from another by ammonium sulfate precipitation, and the hydrophobic protein (HP) fraction (Galante and Hatefi 1979). The FP fraction contains three subunits, the NDUFV1 (51 kDa), NDUFV2 (24 kDa) and NDUFV3 (10 kDa) (Hattori et al. 1995; Schuelke et al. 1998), which contain the NADH-, flavin mononucleotide-, and several Fe-S cluster binding sites. The IP fraction composed of approx. seven subunits, the NDUFS1 (75 kDa), NDUFS2 (49kDa), NDUFS3 (30 kDa), NDUFS4 (18 kDa), NDUFS5 (15 kDa), NDUFS6 (13 kDa) and NDUFA5 (B13) (see Smeitink et al. 1998), which contain several Fe-S cluster binding sites. The FP and IP form the principal catalytic sector and are located in the peripheral arm of the complex (Belogrudov and Hatefi 1994). The HP fraction, which forms a water-insoluble aggregate, contains the seven mitochondrial-encoded subunits and the 16 nuclear-encoded subunits, the NDUFA1 (MWFE), NDUFA2 (B8), NDUFA4 (MLRQ), NDUFA6 (B14), NDUFA8 (PGIV), NDUFA9 (39kDa), NDUFAB1 (SDAP), NDUFB1 (MNLL), NDUFB3 (B12), NDUFB5 (SGDH), NDUFB6 (B17), NDUFB7 (B18), NDUFB9 (B22), NDUFS7 (PSST), NDUFS8 (TYKY) and NDUFC1 (KFYI) (see Smeitink et al. 1998), and is involved in proton translocation (Belogrudov and Hatefi 1994; Ohnishi et al. 1985). Besides hydrophobic subunits, this fraction also contains globular water-soluble subunits. The 51 kDa FP subunit contains the binding sites for NADH (Chen and Guillory 1981; Deng et al. 1990) and the primary electron acceptor, FMN, and also binds a tetranuclear iron-sulfur center (Pilkington et al. 1991). The 24 kDa FP subunit contains a binuclear iron-sulfur center (Pilkington and Walker 1989). The 75 kDa IP subunit contains a tetranuclear and probably also a binuclear iron-sulfur cluster (Runswick et al. 1989). Sequencing of the bovine 23 kDa HP subunit revealed that this subunit contains two cysteine motifs which probably provide the ligands to accommodate two 4Fe-4S clusters (Dupuis et al. 1991). The FP and IP water-soluble fractions make contact through the 51 kDa FP and 75 kDa IP subunits. The FP and IP subunits stoichiometry and the substrate-induced conformational changes have been partly elucidated, where the proximity of the 51 kDa FP subunit to the 75 kDa IP subunit, and the proximity of all studied IP subunits to one another and to some of the HP subunits are altered when the catalytic sector of complex I is reduced by NADH or NADPH prior to the addition of a cross-linking reagent. It has been speculated that these conformational changes may well be the device by which the energy derived from electron transfer through the catalytic components of respiratory complex I is transduced and conveyed to the subunits of the membrane sector (Belogrudov and Hatefi 1994).

The overall function of the respiratory complex I is the dehydrogenation of NADH and the transportation of electrons to coenzyme Q. This electron transport creates, like for respiratory complexes III and IV, a proton-motive force for the production of ATP by F_1-F_o ATP synthase (complex V) (see Fig. 1.1). The activity of the respiratory complex I is inhibited by rotenone, piericidin A, barbiturates, Demerol and mercurials (see Hatefi 1985).

Succinate-ubiquinone oxidoreductase (complex II). The complex II of the mitochondrial respiratory chain is composed of four subunits: two-subunits succinate dehydrogenase (SDH; 70 and 27 kDa) and two polypeptides (15.5 and 13.5 kDa) that contain the cytochrome b_{560} heme (Hatefi and Galante 1980). Treatment with chaotropic agents results only in the water-soluble protein of the SDH components, the 70 kDa ferroflavoprotein (FP) and the 27 kDa iron-protein (IP) subunits. Separation of the two subunits under non-denaturing conditions has shown that the larger polypeptide contains the covalently bound FAD plus four equivalents each of iron and sulfide ions, and the smaller polypeptide contains three iron and three sulfide ions per mole (Davis and Hatefi 1971). This enzyme complex is anchored to the membrane by a b-type cytochrome.

The respiratory complex II is a component of the Krebs cycle which oxidizes succinate to fumarate, and passes the electrons directly into the quinone pool. It serves as the only direct link between activity in the citric acid cycle and electron transport in the membrane. This enzyme complex does not translocate protons, and therefore it only feeds electrons to the electron transport chain (Hagerhall 1997) (Fig. 1.1). The catalytic activity of respiratory complex II can be assayed only in the presence of artificial electron acceptors. All subunits of the respiratory complex II are exclusively coded by the nuclear DNA, and imported from the extramitochondrial cytoplasm. The activity of complex II can be inhibited by 2-thenoyltrifluoroacetone (see Hatefi 1985).

Ubiquinol-cytochrome c *oxidoreductase (cytochrome* bc_1*, complex III).* The respiratory complex III catalyzes electron transfer from ubiquinol to cytochrome *c*, and this redox reaction is coupled to the generation of a proton gradient across the membrane by a mechanism known as the Q cycle (see Fig. 1.1). Ubiquinol is a lipid-soluble compound that can move within the membrane. As the redox chemistry of quinol is coupled to protonation and deprotonation, these two reactions are topologically organized such that the oxidation of quinols leads to active transport of hydrogen ions across the membrane. This requires two active sites, one for the oxidation of ubiquinol and release of protons on the outer surface of membrane (Q_0), and one for the reduction of ubiquinone coupled to the uptake of protons from the inner side of the membrane (Q_1). This mechanism requires that electrons be transferred from the Q_0 site to the Q_1 site (see Hatefi 1985).

The respiratory complex III is located in the middle of the respiratory chain, and is composed of 11 subunits, one of which, the cytochrome *b*, is encoded by the mtDNA, and is a hydrophobic polypeptide of 42.5 kDa. The nuclear-encoded subunits contain two *b*-type and one *c*-type cytochromes, and one iron-sulfur protein. Of the eleven subunits, only three of them carry the redox centers that are used in the conservation of energy (Brandt 1996). The key subunits are: cytochrome *b*, which has eight transmembrane helices with two hemes sandwiched between helices B and D; a membrane-anchored FeS protein (ISP) carrying a Rieske-type center (Fe_2S_2); and a membrane-anchored FeS cytochrome c_1, which is water-soluble and constitutes two different polypeptides of a 241 amino acid and a 78 amino acid residues. Most of the other eight subunits are small proteins that surround the metalloprotein nucleus; two of them face the mitochondrial matrix and are homologous to mitochondrial processing peptidases, which function in protein import (Braun and Schmitz 1993). Thus, the respiratory complex III may be multifunctional. The activity of complex III is inhibited by Antimycin A and myxothiazol.

Cytochrome c oxidase (ferrocytochrome c-oxygen oxidoreductas,; complex IV). The respiratory complex IV is the terminal enzyme of the mitochondrial electron transport system, and located in the inner membrane of the mitochondria. This enzyme complex generates a transmembrane proton gradient (Fig. 1.1). The substrate, cytochrome *c*, is a water-soluble hemoprotein that donates electrons on the cytoplasmic side of the mitochondrial inner membrane. This enzyme contains two hemes and two coppers per monomeric unit, often termed as cytochrome aa_3, which catalyzes the transfer of four electrons from reduced cytochrome *c* via four redox centers (two heme a and two copper atoms) to dioxygen. The protons needed for this reaction are taken from the mitochondrial matrix side through two channels. The same channels are used to pump one proton per electron across the membrane (see Hatefi 1985).

The human cytochrome oxidase contains 13 subunits (Kadenbach et al. 1983), three of which are the major subunits of 56.9, 26 and 29.9 kDa (Tzagoloff et al. 1979), which form the functional core of the enzyme, and are coded for by mtDNA. This core is surrounded by 10 nuclear-coded small subunits. Subunit I contains the active site. Subunit II has a dinuclear, mixed valence copper center (Cu_A) (Lappalainen et al. 1993; Malmstrom and Aasa 1993), which is the first site to receive electron from cytochrome *c*. These electrons are then transferred to cytochrome *a* in subunit I, and then to the bimetallic cytochrome a_3/Cu_B active site (Fig. 1.1). Two hemes are ligated to Cu_B by six histidines. A covalent bond was forms between one of the Cu_B ligands (H420 in the bovine enzyme) with a tyrosine (Y244) from which it is separated by one helical turn

(Tsukihara et al. 1995, Yoshikawa, 1998 #1622). The histidine-tyrosine bond may generate a free radical that plays a role on the reduction of O_2. Subunit III contains bound phospholipids but its functional role has not yet been elucidated. Inhibitors for complex IV include cyanide, azide, and carbon monoxide.

ATP synthase (H+-ATPase, F_O-F_1 ATPase, complex V). The mitochondrial ATP synthase is a functionally reversible enzyme. This enzyme complex is responsible for synthesizing ATP from ADP and inorganic phosphate using a proton-motive force across the membrane derived from the operation of the respiratory complexes I, III, and IV, respectively, and is also capable for hydrolyzing ATP to pump protons against an electrochemical gradient.

The bovine complex V appears to contain 16 distinct subunits (Lutter et al. 1993) and is greater than 500 kDa in size. A membrane sector (F_o) contains the proton channel, and linked to the catalytic component (F_1), located in the matrix side of the membrane, by a stalk consisting of two parallel structures (Bottcher et al. 1998) referred to as "rotor" and "stator" (Elston et al. 1998). Two subunits of the respiratory complex V, subunit 6 and 8, are mitochondrial-encoded, and belong to the F_0 sector. The soluble F1 sector can be detached from the complex, and consists of five subunits: α, β, γ, δ, and ε, in a stoichiometry 3:3:1:1:1, and forms a spherical globular structure. The α and β subunits are homologous; both bind nucleotides, but only β has catalytic activity. Thus, there are three active sites within the catalytic component; each site would pass through a cycle of three different stated ("open", "loose", and "tight", corresponding to an empty state, a state with bound ADP and phosphate, and a state with tightly bound ATP), and at any given moment, the three sites would be in a different state (Boyer 1993, 1997 #1633). The formation of ATP does not require energy once the substrates have been separated from the aqueous solution; energy is required for substrate binding and the release of ATP (Boyer 1993; Boyer 1997). The synthesizing and hydrolyzing activities of complex V can be inhibited by oligomycin and dicyclohexylcarbodiimide (DCCD) which block the proton channel of F_0, as well as uncouplers 2,4-dinitrophenol (DNP) and carbonylcyanide-p-trifluoromethoxyphenyl–hydrazone (FCCP).

The Mitochondrial Genome

Animal mitochondrial genomes are normally circular, ~16 kb in length, located in the matrix of the double-membrane mitochondrion, and encode proteins, which are subunits of the respiratory enzyme complexes responsible for energy production, as well as tRNAs and rRNAs. The genome contains a non coding region (the displacement (D) loop region), which is known as well as the control region, where origin of replication, initiation of replication and transcription are located.

The human mitochondrial DNA (mtDNA) is a circular, double stranded 16,569-base pair DNA containing very few non coding regions and several overlapping coding regions. It has an extremely efficient and compact organization in the heavy and light strands (Anderson et al. 1981).

Human mtDNA encodes 13 polvpeptides, subunits of respiratory enzyme complexes; seven subunits of the NADH-ubiquinone oxidoreductase (ND1, ND2, ND3, ND4, ND4L, ND5, and ND6), one subunit of the ubiquinol-cytochrome *c* oxidoreductase (apocytochrome b), three subunits of the cytochrome *c* oxidase (COI, COII, and COIII), and two subunits of the ATP synthase (ATP6 and ATP8) (Anderson et al. 1981; Chomyn et al. 1985; Tzagoloff and Myers 1986). MtDNA also encodes for the small (12S) and large (16S) rRNA, as well as for 22 distinct tRNAs, all of these are components of mitochondrial protein synthesizing machinery. The heavy (H)-strand harbours most of the guanine (G) residues, and functions as the template for the 12S and 16S rRNAS, 12 of the polypeptides, and 14 of the tRNAs. The light (L)-strand contains most of the cytosines (C) residues, and is the template for one polypeptide (ND6) and 8 tRNAs (Fig. 1.2) (Wallace 1991).

The Control (Displacement [D]-Loop, Non Coding) Region

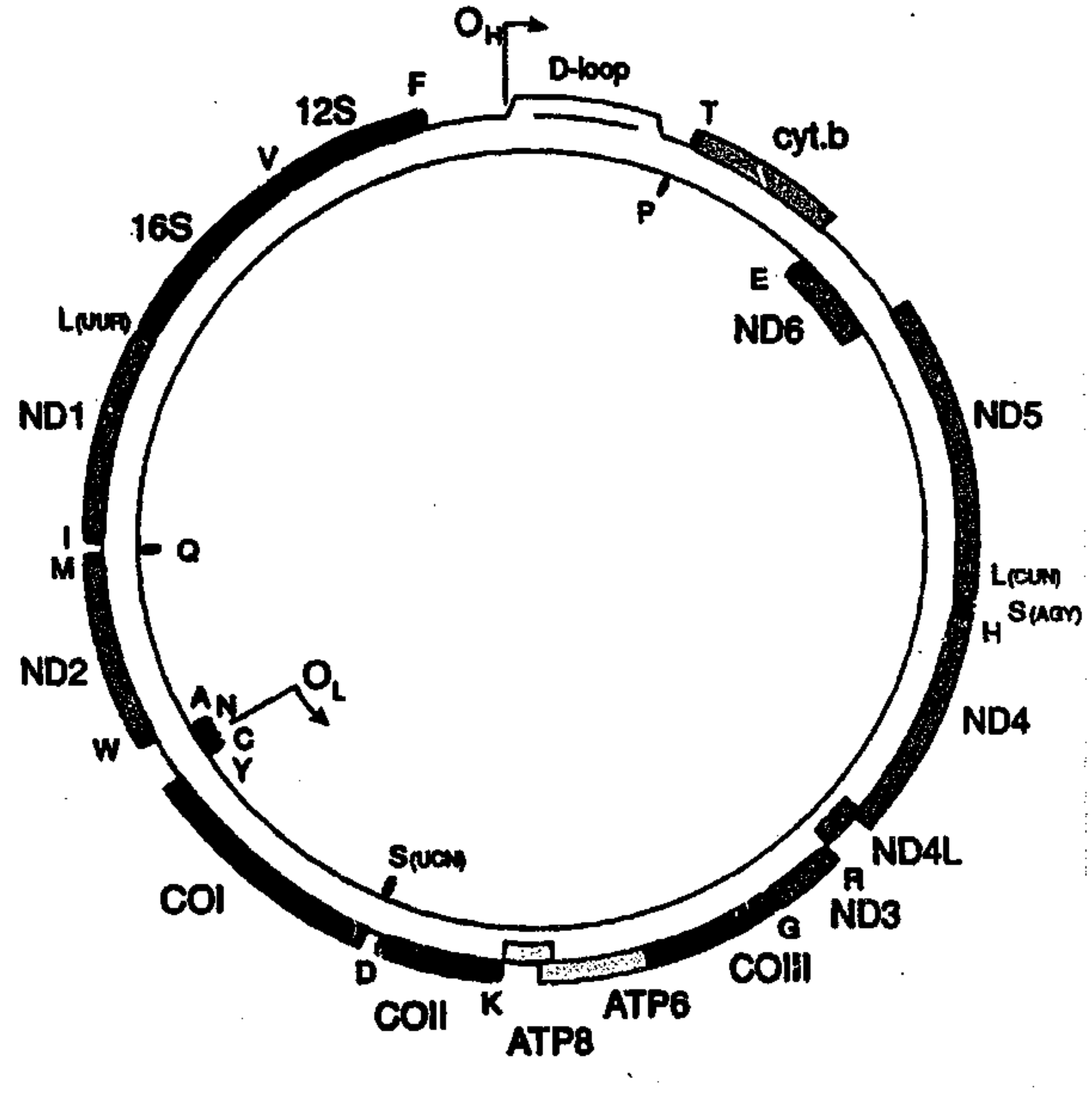
In mammalian cells that are metabolically active, a large proportion of the mtDNA duplexes contain a short three-stranded structure, which is called the displacement loop or D-loop (Kasamatsu et al. 1974); a short nucleic acid strand, complementary to the L-strand, that displaces the H-strand.

The D-loop region is flanked by the genes for $tRNA^{Phe}$ and $tRNA^{Pro}$ (Walberg and Clayton 1981) (see Fig. 1.2), and has evolved as the major control site for mtDNA expression. The D-loop contains: the leading (H)-strand origin of replication (O_H), the two mtDNA promoters, the H-strand and the L-strand promoters (HSP and LSP); the two major transcription initiation sites, the H-strand initiation site (IT_{H1}), located within the HSP, and L-strand transcription initiation site, located in the LSP (IT_L), both are separated 150 bp one from another; three conserved sequence blocks (CSBI, CSB II, and CSB III), and the termination associated sequence (TAS) as illustrated in Fig. 1.3 (see Taanman 1999).

Sequences from the mtDNA D-loop region, which spans nucleotides 16028-16569 and 1-577 (numbered according to the Cambridge Reference Sequence (CRS), Anderson et al. 1981), are highly variable within human populations. The highest degree of polymorphism lies within two hypervariable segments of the non-coding region, the hypervariable region I (HVRI) and HVRII. Sequence information from the mtDNA control region, which documents variation within and between human populations, has been widely used to infer certain aspects of human population and demographic history (see Cann et al. 1987; Vigilant et al. 1991; Stoneking et al. 1992; Horai 1995). The use of sequence

Figure 1.2. The Human Mitochondrial Map.

The human mitochondrial DNA (mtDNA) is a circular, double stranded 16,569-base pair, encoding 13 subunits of respiratory enzyme complexes. Those are seven subunits of the NADH-ubiquinone oxidoreductase are ND1, ND2, ND3, ND4, ND4L, ND5, and ND6, one subunit of the ubiquinol-cytochrome *c* oxidoreductase (apocytochrome b), three subunits of the cytochrome *c* oxidase (COI, COII, and COIII), and two subunits of the ATP synthase (ATP6 and ATP8) (Anderson et al. 1981; Tzagoloff and Myers 1986). MtDNA also encodes for the small (12S) and large (16S) rRNA, and also for 22 distinct tRNAs. Except for subunit ND6 of NADH-ubiquinone oxidoreductase and eight tRNAs, which are contained in the light (L)-strand, the other polypeptides, the two rRNAS and the rest of the tRNAs are encoded in the heavy (H)-strand.


O_H
D-loop
F
12S
T
cyt.b
V
P
16S
E
ND6
L(UUR)
ND1
ND5
I
Q
M
L(CUN)
S(AGY)
H
ND2
O_L
A
N
C
Y
W
ND4
ND4L
S(UCN)
R
ND3
COI
G
COIII
D
COII
K
ATP6
ATP8

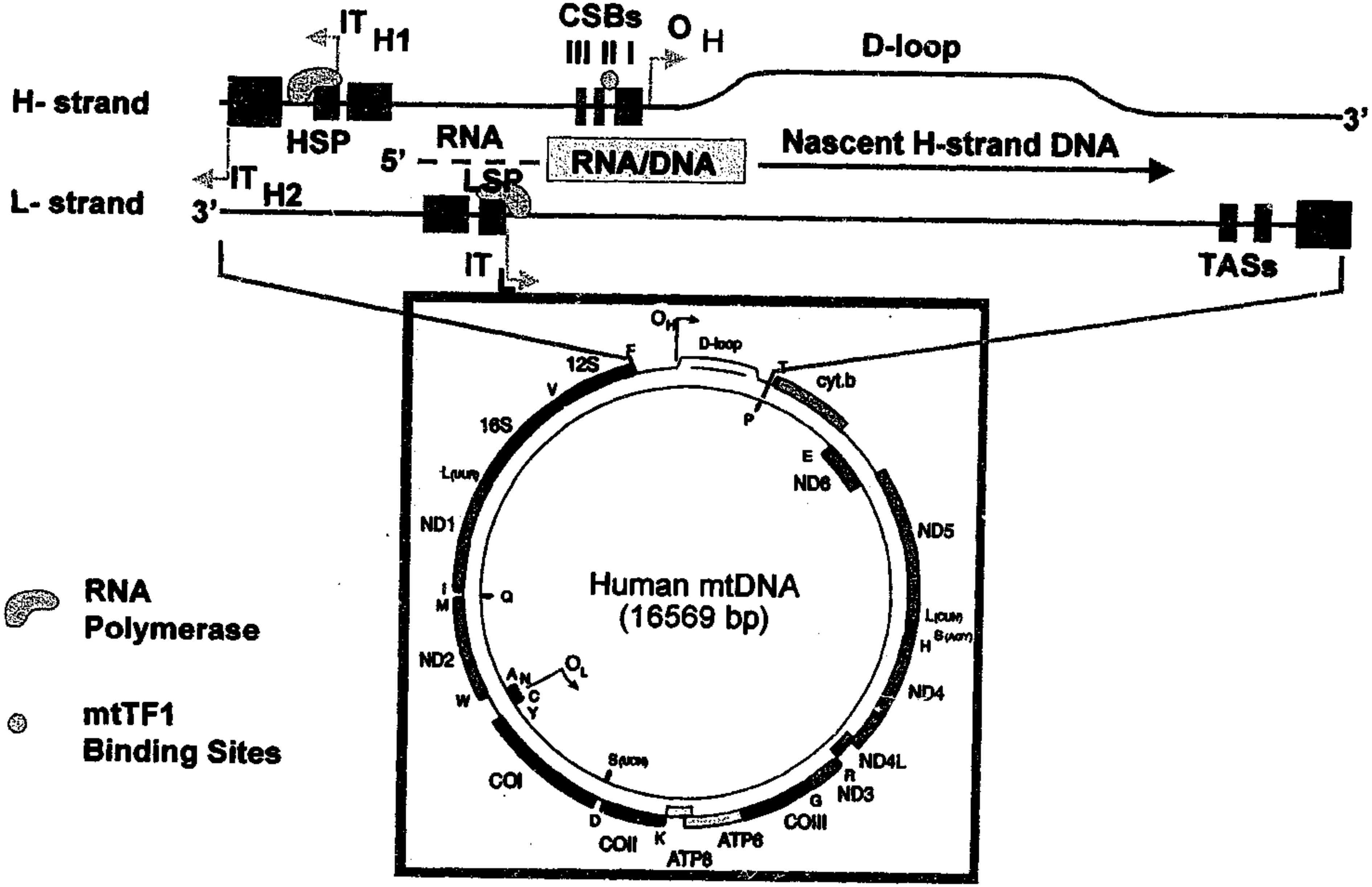
information from the control region to assess the time elapsed since a particular event took place requires knowledge of the mutation rate in this region, since it is apparent that certain sites in the mtDNA have been found to be "hotspots" for mutations (Hasegawa et al. 1993; Wakeley 1993; Excoffier and Yang 1999; Meyer et al. 1999; Stoneking 2000), and it is known that mutations in the HVRII have a higher heterogeneity of rates than those in the HVRI (Aris-Brosou and Excoffier 1996).

Inheritance Characteristic and Random Segregation

Mitochondrial DNA is transmitted exclusively through the maternal line. The distribution of mitochondria to daughter cells during cell division is an essential feature of cell proliferation. Until recently, it was commonly believed that inheritance of mitochondria was a passive process, a consequence of their random diffusion throughout the cytoplasm. Insights into the molecular basis of mitochondrial behavior came from a combination of microscopic, genetic, and biochemical approaches. In the yeast *S. cerevisiae*, which proliferates by a budding process, a mother cell produces a daughter bud that grows larger and eventually becomes an independent cell. During that process, there is a vectorial transport of mitochondria and other organelles into the developing bud (Simon et al. 1997). The analysis of yeast mutants defective in this transport process

Figure 1.3. Anatomy of the Mitochondrial DNA D-Loop Regulatory Region.

Schematic representation of the major features of the human mtDNA D-loop, showing the site of initiation of transcription and replication. The genes coding for $tRNA^{Phe}$ (F) and $tRNA^{Pro}$ (P) are indicated with boxes at the H- and L-strands. Transcription initiation sites (IT_{H1}, IT_{H2}, and IT_{L}) and direction of synthesis are indicated by bent arrows; the dashed/solid line represents an RNA-primed nascent DNA strand, where the transition from RNA to DNA synthesis is indicated with box, around the conserved sequence blocks (CSBs) I, II and III. O_H is the origin of replication for the H-strand. The non-expanding small D-loop strand, which is part of the triplex D-loop structure terminate near the termination-associated sequence (TAS). Mitochondrial RNA polymerase is shown at the transcriptional start site at the L-strand promoter (LSP) and H-strand promoter (HSP). The 16,569 bp circular human mitochondrial DNA (mtDNA) is shown at the bottom panel.


IT
H1
CSBs
III II I
O
H
D-loop
H- strand
HSP
RNA
5'
RNA/DNA
Nascent H-strand DNA
3'
IT
H2
LSP
L- strand
3'
IT
TASs
O
H
D-loop
12S
F
T
cyt.b
P
V
16S
E
ND6
L(UUR)
ND5
ND1
Human mtDNA
(16569 bp)
I
M
Q
L(CUN)
S(AGY)
H
ND2
A
N
C
Y
O
L
ND4
W
ND4L
S(UCN)
R
ND3
COI
G
COIII
D
K
ATP6
COII
ATP8
RNA Polymerase
mtTF1 Binding Sites

has led to the identification of proteins that facilitate mitochondrial inheritance. The first mitochondrial inheritance affected mutants were isolated by screening microscopically collections of temperature-sensitive strains for cells that failed to transport mitochondria into daughter buds. Buds of these mitochondria devoid mutants never became viable cells, reflecting the essential requirement for mitochondria (McConnell et al. 1990)

Family studies using RFLPs as mtDNA genetic markers show that human mtDNA is transmitted from the mother to all of her offspring and from her daughters to the next generation, but males do not transmit their mtDNA (Giles et al. 1980; Case and Wallace 1981). When a mutation arises in one of the mtDNA within a cell, this creates an intracellular mixture of mutant and wild type molecules, known as heteroplasmy. When a heteroplasmic cell divides, it is a matter of chance which mtDNAs will be partitioned into the daughter cells. As a result, over many cell divisions the proportion of mutant and normal mtDNAs can drift toward either pure mutant or pure wild type (homoplasmy) (Hauswirth and Laipis 1982). The drift could also be a consequence of "relaxed" replication in which some molecules are not copied, while others act as templates for several replication events. Evidence has been recently demonstrated that in cultured HeLa and neuronal PC12 cells, the replication of mtDNA molecules appeared to occur preferentially in close proximity to the nucleus, sparing the peripheral mtDNA (Davis and Clayton 1996). One of the most controversial examples of relaxed transmission and replication has been termed the mtDNA "bottleneck" and is implied to occur during oogenesis (for review see Lightowlers et al. 1997). The mtDNA bottleneck is actually a series of events during oogenesis where mtDNA molecules are selectively sampled from a larger population, restricting the numbers of mtDNA to be transmitted, followed by amplification. Eventually, only a small number of mtDNAs populate the organism.

Replication of the Mitochondrial DNA and Its Regulation

The mode of replication of the mtDNA in animal cells grown in culture (Robberson et al. 1972) has been established by ordering the various replicative intermediates seen in the electron microscope. Early studies indicated that mammalian mtDNA molecules replicated unidirectionally from two spatially and temporally distinct, strand-specific origins (Robberson and Clayton 1972; Kasamatsu and Vinograd 1973; Kasamatsu and Vinograd 1974; Clayton 1987). The origin of H-strand replication (O_H) is located downstream of the Light strand promoter (LSP) in the D-loop region of the genome (Fig. 1.3), whereas the origin of L-strand replication (O_L) is at two-thirds of the genomic distance away from O_H with respect to the polarity of H-strand synthesis. A round of replication begins at the O_H in the D-loop region, where short transcripts from an upstream promoter serve as the primers for the synthesis of nascent H-strands. The transition from RNA to DNA synthesis occurs at several distinct sites that collectively constitute O_H in a region of three short, evolutionary conserved sequence blocks, named CSB I, II and III, and is mediated by mitochondrial RNA processing endonuclease (Rnase MRP specific) cleavage of the primary transcript (for review see Clayton 1987; Rahman et al. 1999; Shadel and Clayton 1997). The non-expanding small D-loop served as a marker for the expansion that occurs in replication. A round replication begins at O_H with the synthesis of a daughter H-strand and continues along the parental L-strand to produce a full H-strand circle. Only after the replication fork has passed the O_L, synthesis of the L-strand is initiated, which proceeds in a direction opposite to that of H-strand replication (for review see Taanman 1999; Shadel and Clayton 1997). Thus, mtDNA replication is bidirectional but asynchronous.

Despite the asymmetry, the mtDNA replication is semiconservative. A unique feature of mtDNA replication is that displacement synthesis temporarily terminates after a small percentage of the genome has been replicated to form D-loop DNA (Kasamatsu et al. 1971), which accumulates awaiting some signal for the extension.

The replication process involves several proteins, which has been identified, including: (a) DNA polymerase γ, the only DNA polymerase present in mitochondria (Bolden et al. 1977), which has been purified in (Gray and Wong 1992), and consist of two subunits, the 140 kDa catalytic subunit (Ropp and Copeland 1996) containing DNA polymerase (Ropp and Copeland 1996; Foury 1989; Ropp and Copeland 1995), 3'→5' exonuclease (Gray and Wong 1992; Ropp and Copeland 1996; Foury and

Vanderstraeten 1992; Insdorf and Bogenhagen 1989; Kaguni and Olson 1989; Lecrenier et al. 1997), and 5' dRP lyase activities (Longley et al. 1998; Longley et al. 1998), and the 55 kDa accessory subunit (Lim et al. 1999), which has a role as a processivity factor and implies it improves the fidelity of DNA synthesis; (b) a ribonucleoprotein, mtDNA-processing endoRNase (Rnase-MRP), the RNA component of which is encoded by a nuclear gene (Chang and Clayton 1989); (c) the yeast mtDNA helicase (Foury and Lahaye 1987); and (d). a single stranded (ss) DNA-binding protein, which improves the mitochondria replication system by: (i). improving the polymerization efficiency by preventing the formation of secondary ssDNA structures (Van Tuyle and Pavco 1981); Mignotte et al. 1988), and (ii). improving the proofreading capability by enhancing the mismatch-specific 3'→5' exonucleolytic activity (Gray and Wong 1992).

Transcription of the Mitochondrial DNA

Direct evidence of the location of the mammalian mtDNA transcription promoters have been obtained, and the mechanism of mitochondrial transcription have been solved (for review see Taanman 1999; Clayton 1984; 1991). The transcription initiation sites and promoter regions have been identified, with all data consistent revealing that there are two major transcription initiation sites in the D-loop, known as IT_{H1}, the initiation site for the H-strand, and $IT_{L,}$ the initiation site for the L-strand, located approx. 150 bp away one from another (Fig. 1.3). Each of the initiation site is surrounded by a promoter element (H-strand promoter/HSP and L-strand promoter/LSP) with a 15-bp consensus sequence motif, although there is no sequence conservation between species (Rahman et al. 1999; Clayton 1991). Enhancer elements, including the binding sites of mitchondrial transciption factor A (mTFA) (Fisher et al. 1987); which located upstream of the promoters are required for optimal transcription. The binding of mTFA to this region is required for specific initiation of transcription (Fisher et al. 1987; Fisher and Clayton 1988; Topper and Clayton 1989).

The HSP and LSP are known to be functionally independent, despite their closeness, as demonstrated in muscle fibers of patients with large scale mtDNA deletion, where focal accumulations of deleted mtDNA and L-strand transcripts with concomitant depletion of H-strand transcripts was observed (Moraes et al. 1991; Hammans et al. 1992).

There is another initiation site for the H-strand (IT_{H2}), which is located adjacent to the 12S rRNA gene (Fig. 1.3). However, this site is not used as frequently as IT_{H1} (Chang and Clayton 1984).

The transcript of the L-strand elongated as a single polycystronic precursor RNA once the initiation started at the LSP (Aloni and Attardi 1971; Murphy et al. 1975). HSP-directed transcription started more frequently at the IT_{H1}, and terminated at the downstream end of the 16S rRNA gene; this process is responsible for synthesis of the majority of the 12S and 16S rRNA species. Transcription started at IT_{H2}, which is less frequent, resulted in polycycstronic molecules corresponding to almost the entire H-strand, containing all the mRNAs and tRNAs encoded in the H-strand (Montoya et al. 1982). The mature mRNAs, rRNAs and tRNAs are then released by cleavage (Clayton 1992).

Translation of the mtDNA Transcript

The spectrum of antibiotics that inhibits mitochondrial protein synthesis is similar to that of prokaryote protein synthesis, which indicated the prokaryotic origin of mitochondria (for review see Borst and Grivell 1981). The initiation of the translation process in mammalian mitochondria is unique, unlike prokaryotic and eukaryotic cytosolic messengers, the mammalian mitochondrial mRNAs does not have upstream leader sequences, which is needed to facilitate ribosome binding. The process is started at or very near 5'-end with the codon for the initiating *N*-formylmethionine (Montoya et al. 1981).

Indication of the ability of the small (28S) ribosomal subunit to bind mRNA tightly in a sequence independent manner and in the absence of additional initiation factors or initiator tRNA was obtained by *in vitro* experiment with bovine mitochondrial ribosomes (mitoribosomes) (Liao and Spremulli 1989). After the small ribosomal subunit binds to mRNA, it is assumed that the subunit will move to the 5'-end of the mRNA. However, the additional initiation factors that mediated this process has not been specified yet (Liao and Spremulli 1990), except the mtIF-2 (Liao and Spremulli 1990); Liao, 1991). The human mtIF-2 shows 39% amino acid homology to that of *E. coli* (Ma and Spremulli 1995). The mtIF-2 in *E. coli* is known to promotes fMet-tRNA binding to the small ribosomal subunit in the presence of GTP and a template (Ma and Spremulli 1995).

The mitochondrial elongation factors have been identified and purified from bovine liver, known as mtEF-Tu, mtEF-Ts and mtEF-G (Schwartzbach and Spremulli 1989; Chung and Spremulli 1990) Based on *in vitro* characterization of the purified factors and the cDNA sequence information, similarities of these factors with the corresponding prokaryotic factors have been revealed. Thus, it is assumed that the elongation process of the nascent mitochondrial polypeptide proceed in a similar manner as in *E. coli* (see Nierhaus 1996).

NUCLEAR AND MITOCHONDRIAL GENOMES CROSSTALK

Nuclear-Mitochondrial Interactions

Only a few of the hundred or so mitochondrial proteins are encoded by the mtDNA. The remainder, about 90% of the proteins that exist in the mammalian mitochondrion including most of the components of the mitochondrion's own genetic system, which also specify other enzymes necessary for the synthesis, import, processing and modification of these proteins and associated lipid components, are encoded by the nuclear genes and translated in the cytoplasm, and subsequently transported posttranslationally into the organelle (for review see Glick and Schatz 1991). The assembly of a functional mitochondrion in eukaryotic cell is possibly one of the most complex process resulting from a joint effort between gene products of both mitochondrial and nuclear genomes, requiring a significant level of nucleo-mitochondrial interaction (see Grivell 1995; Attardi and Schatz 1988). The nuclear and mitochondrial genomes interact in at least two ways: first, both nuclear and mitochondrial genes donate essential subunit polypeptides to mitochondrial respiratory function; second, both nuclear and mitochondrial genomes collaborate in the synthesis and assembly of mitochondrial proteins. The first interaction is important for the regulation of oxidative energy production. The second interaction requires the bidirectional flow of information between the nucleus and the mitochondrion. Communication from the nuclear genome to the mitochondrion utilizes proteins that are translated in the cytoplasm and imported into the mitochondrion, requiring the machinery of mitochondrial protein import. On the other hand, communication from the mitochondrion to the nucleus is thought to involve metabolic signals and signal transduction pathways, which function across the inner mitochondrial membrane (Poyton and McEwen 1996).

Import of Cytoplasmically Synthesized Proteins into Mitochondria

The polypeptides encoded by nuclear and mitochondrial genes follow two different pathways to reach their destination within the mitochondrion. Cytoplasmically synthesized mitochondrial proteins nuclear-encoded mitochondrial proteins are synthesized in the cytosol on cytosolic ribosomes as precursors, and imported into mitochondria after the synthesis of the precursor chain has been compelted. Cytoplasmically synthesized proteins that are destined to be imported into mitochondria have to go through a process of four distinct steps: (1) mitochondrial targeting, (2) protein unfolding, (3) sorting of the polypeptides to either routed to their correct submitochondrial compartement, or transported across two membranes, depends on the properties and final destination of each protein, and (4) polypeptide maturation (see Neupert 1997; Pfanner et al. 1997; Schatz 1996 for review).

Nuclearly encoded proteins are normally synthesized with a positively charged N-terminal extension as a matrix-targeting signal, which will be removed once the protein has reached its correct intramitochondrial destination (for review see Schatz 1996). Subsequently, these precursor proteins are imported into the mitochondria in a multistep process by translocation systems in the mitochondrial outer and inner membranes. Three hetero-oligomeric protein translocation channels have been identified, the translocase of the outer membrane (TOM) and two translocases of the inner membrane (TIM), TIM23 and TIM22, complexes (see Neupert 1997; Pfanner et al. 1997; Schatz and Dobberstein 1996). A more general precursor proteins with a positively charged N-terminal presequence are imported via the TIM 23 complex, while the TIM22 complex, an integral inner membrane protein of mitochondria, mediates the import of carrier proteins that do not carry the matrix-targeting signal, (see Bauer et al. 2000 for recent review).

Protein import into the matrix space requires both an electrochemical potential $\Delta\psi$ across the inner mitochondrial membrane and the function of the mitochondrial hsp70 (mhsp70) chaperone (see Neupert 1997), which interact with the TIM44, a peripheral membrane protein associated with the inner face of the inner mitochondrial membrane in an ATP-dependent manner (Krimmer et al. 2000; Horst et al. 1996). Recent evidence suggests that mhsp70 is a mechanochemical enzyme that actively pulls precursors across the inner membrane (Horst et al. 1997). Precursor proteins are imported as an unfolded protein during translocation, and threaded through the protein import machinery as linear chains. Both the $\Delta\psi$ and the mhsp 70 contribute to protein unfolding (for review see Matouschek et al. 2000).

Once the precursor protein has reached the mitochondria matrix, the positively charged N-terminal targeting sequence is usually removed by proteolytic enzymes, and the protein refolds into its native structure (for review see Matouschek et al. 2000).

Proteins encoded by mitochondrial genes are translated on endogenous mitochondrial ribosomes that are bound to the matrix side of the inner membrane, and they are inserted into the inner membrane from within cotranslationally (see Attardi and Schatz 1988) for review.

Nuclearly Coded Subunits of the Mitochondrial Respiratory Chain

The nuclearly coded mitochondrial proteins play important roles in all aspects of mitochondrial function, including the tissue specific expression of the oxidative phosphorylation (OXPHOS). Evidence of tissue specific isoenzyme forms of the respiratory chain complexes has been obtained (Capaldi et al. 1988). It was postulated that some of the nuclear-encoded subunits of cytochrome *c* oxidase are involved in allosteric modification of the enzyme (Kadenbach and Merle 1981). Nuclearly coded tissue-specific subunits of mammalian cytochrome oxidase has been demonstrated for muscle tissue (Arnaudo et al. 1992). An illustration of tissue specific isoform which reflects the differential dependence of certain tissues on oxidative phosphorylation is the tissue-specific expression of the adenine nucleotide translocator (ANT) gene family. The ANT catalyzes the exchange of ADP and ATP across the mitochondrial inner membrane. Three isoforms of the ANT have been identified and known to be coded by differentially regulated nuclear genes (Stepien et al. 1992). High expression of the ANT1 is found in heart and skeletal muscle; the expression is induced during myoblast differentiation. The expression of the ANT2 is either weak or absent in all tissues. The ANT3 is ubiquitously expressed in all tissues; the transcript level of this isoform is proportional to the level of oxidative metabolism (Stepien et al. 1992).

Many mitochondria associated diseases have been demonstrated to show tissue specific expression. The examples include the Leber's hereditary optic neuropathy (LHON), which the clinical manifestation occurs mainly in the optic nerve (Wallace et al. 1988), the aminoglycoside induced deafness associated with the homoplasmic mtDNA A1555G mutation in the 12S rRNA gene, which the clinical defect remains confined to the cochlea (see Fischel-Ghodsian 1998, for review).

MITOCHONDRIAL DNA MUTATIONS AND DISEASES

The accumulation of mtDNA mutations during life is much more rapid than in nuclear genes because of (a) the lack of a major repair mechanism in the genome, (b) the high generation of oxygen radical in the organelle as the side product of mitochondrial electron transport chain, and (c) the absence of protective histones. Many of the mtDNA mutations are inherited through maternal line, but others occur sporadically and are presumably caused by spontaneous mutations early in the development, or even in the unfertilized oocyte. As noted in the beginning of this chapter, mutations in the mtDNA, either a single nucleotide mutation or a large deletion, have been shown to be associated with a diverse spectrum of human disorders. Mitochondrial defects do not only occur in a wide variety of degenerative diseases and aging, but also in cancer.

Biochemical Defects and Clinical Manifestations Related to Mitochondrial Disorders

MtDNA diseases commonly have a delayed onset and a progressive course. Enzymatic biochemical disorders consequential of a mtDNA mutation and their clinical manifestations appear to be the outcome of an interplay between the causal mutation and other sequence variants of the mtDNA, and between the nuclear and the mitochondrial genetic systems (see Wallace 1999; Hanna and Nelson 1999; Zeviani and Antozzi 1997). The expression of pathological mtDNA mutations is determined and modulated also by random cellular events during development. MtDNA carrying a pathological mutation often co-exists in cells with the normal mtDNA (mtDNA heteroplasmy), and the two populations of mtDNA segregate randomly during cell division resulting in varying degree of heteroplasmicity in different cells. The age-related somatic accumulation of mtDNA mutations proposed to be an important factor in the ageing process in which a decline in mitochondrial bioenergetic function in various tissues is observed (Linnane et al. 1989; Trounce et al. 1989) is also a random cellular event. It has been reported recently that each respiratory enzyme complex activity can vary according to the tissues, thus the sensitivity of a particular enzyme complex to a defect as a consequence of a mtDNA mutation also varies (biochemical threshold) (Rossignol et al. 1999). Thus, the adult onset manifestation of most pathological mtDNA mutations has been suggested to be the result of a combination of the two factors mentioned above; (i) the degree of heteroplasmicity, or (ii) the expression of these mutations against a background of an age-related decline in the tissue capacity for oxidative metabolism. The respiratory enzyme deficiencies observed in tissues as the consequence of pathological mtDNA mutations (Collins et al. 1991; Keightley et al. 1996 for examples), and that observed in ageing tissues (see Muller Hocker 1990), are in general of a mosaic nature. In the beginning, inherited mtDNA mutations affect mitochondrial function only subtly, allowing tissues throughout the body to produce the energy they need, at least for a certain time. As the respiratory chain participates in energy production, the oxygen free radicals are being generated as the toxic-by products of the process. These oxygen derivatives carry an unpaired electron, and therefore are highly reactive. They can cause damage to all components of living cells, including respiratory chain proteins and mtDNA. Anything that disrupts the flow of electrons through the respiratory chain, including a single mtDNA mutation, would increase the electrons transfer to oxygen molecules and promote the generation of free radicals, leading to more mtDNA mutations. The accumulation of random somatic mutations in the course of a lifetime depresses further energy production, until eventually a given tissue's energy level falls too low to allow normal operations to continue. Then the tissue begins to perform improperly, and symptoms start to emerge. As somatic mutations accumulate further, energy output continues to decline, and symptoms progress.

The phenotypes or clinical manifestations of mitochondrial diseases are variable; they can be both diverse and overlapping, ranging from single-organ involvement to severe multisystem disease. The same mtDNA mutation may present with very different phenotypes, and different mutations may cause similar phenotypes. There are several factors that influence the variability in clinical manifestation; (1) the ratio of wild-type to mutant mtDNA, (2) varying thresholds of biochemical expression for both the mutation and the tissue involved, and (3) the modulating effect of nuclear and other mitochondrial genes.

Diseases Related to Mutations Affecting Protein Coding Genes

Leber's Hereditary Optic Neuropathy (LHON). LHON is a form of midlife onset, maternally inherited degenerative disorder, characterized by visual failure resulting from a bilateral optic atrophy, with multiple organ involvement in certain families. The relationship between the mtDNA mutations associated with LHON, the resulting biochemical defects and its clinical manifestation, however, is still not well understood and appears to be complex. Only a portion of the maternal relatives, commonly male members of the pedigree, looses their vision (see Wallace 1992; Shoffner and Wallace 1994). The incomplete penetrance offers strong evidence that there must be additional factors, genetic or environmental or both, that modulate the pathogenic phenotypes.

The mtDNA mutations identified to be associated with LHON are divided into two groups, the primary (Table 1.2) and secondary (Table 1.3) mutations. Five appear to play a primary role in causing the disease (Table 1.2; (Brown and Wallace 1994)). The most common cause and most severe of LHON mutation is the G11778A mutation in the ND4 gene (Wallace et al. 1988). Around 50-70% of LHON pedigrees carry this mutation (see Wallace, 1999). The next most common cause but less severe mutation of LHON is a G to A mutation in the ND1 gene at nt 3460 (Howell et al. 1991; Huoponen et al. 1991). A milder, but comparably frequent mutation is the T14484C mutation in the ND6 gene (Johns et al. 1992). Those are the most frequent mtDNA mutations found to be associated with LHON. A T to C mutation at nt 4160 in the ND1 gene is also one of the primary LHON mutation (Howell et al. 1991). The mildest of the primary LHON mutations is the G15257A in the cytb gene (Brown et al. 1992; Johns and Neufeld 1991). The T14484C mutation and the G15257A of LHON mutations are frequently found together (Wallace 1994). The G14459A mutation in the ND6 gene of the mtDNA is included in the primary LHON mutation because it is by far the most severe of the LHON mutations, with two very different clinical phenotypes observed in maternal relatives; one was LHON, the other involved more severe neurological symptoms including early-onset generalized dystonia (Jun et al. 1994). Several secondary mutations associated with LHON have been identified. These mutations by themselves do not manifest in LHON phenotypes, but they seem to act synergistically by increasing the probability of blindness (Table 1.3).

NARP and Leigh syndrome. A T to G or T to C base substitution at nt 8993 in the *ATP6* gene, encoding subunit 6 of mitochondrial ATP synthase, converts the highly conserved leucine 156 to an arginine (Holt et al. 1990; Tatuch et al. 1994). This mutation is invariably heteroplasmic; when the mutant mtDNAs present in a small percentage (<75%), it can cause neurogenic muscle weakness, ataxia, and retinitus

Table 1.2. Primary LHON mutations

MtDNA Mutation	Gene	Amino Acid Change	References
G3460A	ND1	Moderately conserved A 52 to T	(Howell et al. 1991; Huoponen et al. 1991)
T4160C	ND1	Highly conserved L 285 to P	(Howell et al. 1991)
G11778A	ND4	Highly conserved R 340 to H	(Wallace et al. 1988)
G14459A	ND6	Highly conserved A 72 to V	(Jun et al. 1994)
T14484C	ND6	Mildly conserved M 63 to V	(Johns et al. 1992)
G15257A	cytb	Highly conserved D 72 to N	(Brown et al. 1992; Johns and Neufeld 1991)

pigmentosa (NARP). However, when present in a higher percentage (>95%), it can cause Leigh's syndrome, a frequently lethal childhood disease associated with the progressive degeneration of the basal ganglia (Tatuch et al. 1994; Ortiz et al. 1993), characterized by heterogenous disturbances, including deficiencies in pyruvate dehydrogenase complex, cytochrome oxidase, and NADH-coQ reductase (Pastores et al. 1994; Rahman et al. 2001).

The T8993G mutation causes a block in the F_o proton channel of the ATP synthase (Tatuch and Robinson 1993). The ATP synthase defect and the variable clinical phenotypes correlate with the proportion of wild-type/mutant mtDNA corresponding to the T8993G mutation (Trounce et al. 1994)

Diseases Associated with Mutations Affecting Mitochondrial Protein Synthesis

Myoclonic Epilepsy and Ragged Red Fibers (MERRF). The MERRF syndrome is inherited maternally, characterized by myoclonic epilepsy, cerebellar ataxia, and progressive muscular weakness. The generation of ragged red fibers is an indication of proliferation and subsarcolemmal accumulation of mitochondria. The MERRF syndrome is most commonly

Table 1.3. Secondary LHON mutations.

MtDNA Mutation	Gene	Amino Acid Change	References
G3316A	ND1	A→T	(Matsumoto et al. 1999)
T3394C	ND1	Y→T	(Matsumoto et al. 1999)
G3496T	ND1	A→S	(Matsumoto et al. 1999)
C3497T	ND1	A→V	(Matsumoto et al. 1999)
A4136G	ND1	Y→C	(Howell et al. 1991)
T4216C	ND1	Y→H	(Johns and Berman 1991)
A4917G	ND2	D→N	(Johns and Berman 1991)
G5244A	ND2	G→S	(Brown et al. 1992; Matsumoto et al. 1999)
G7444A	COI	Ter→K	(Brown et al. 1992)
T9101C	ATP6	I→T	(Lamminen et al. 1995)
G9438A	COIII	G→S	(Matsumoto et al. 1999; Johns and Neufeld 1993)
G9738T	COIII	A→T	(Johns and Neufeld 1993)
G9804A	COIII	A→T	(Matsumoto et al. 1999; Johns and Neufeld 1993)
T10663C	ND4L	V→A	(Brown et al. 1995)
G13730A	ND5	G→S	(Howell et al. 1993)
C14568T	ND6	G→E	(Wissinger et al. 1997)
G15812A	cytb	G→S	(Brown et al. 1992; Johns and Neufeld 1991)

the result of an A to G base substitution at position 8344 in the mitochondrial tRNA^{Lys} gene, which alters the TΨC loop of tRNA^{Lys} (Shoffner et al. 1990; Yoneda et al. 1990; Noer et al. 1991; Hammans et al. 1993). Like many deleterious tRNA mutations, the 8344 tRNA^{Lys} mutation occurs in a heteroplasmic manner. The proportion of mutant mtDNA varies among individuals within a MERRF maternal lineage, and even among the tissues of the same individual (Chinnery et al. 1997; Lertrit et al. 1992). The level of mutant mtDNA correlates with age of onset and clinical severity of the disease (Hammans et al. 1993; Chinnery et al. 1997) the clinical symptoms usually presents in late adolescence or early adulthood.

The A8344G mutation causes a functional defect in mitochondrial protein synthesis; abnormal polypeptides of mitochondrial translation products were observed (Noer et al. 1991; Seibel et al. 1991; Chomyn et al. 1994). It was suggested that the defective protein synthesis in MERRF is a result from a decrease in tRNA^{lys} aminoacylation capacity, which leads to premature termination of translation at each lysine codon, and accounts for the preferential reduction in Complex I and IV, the complexes with the most mitochondrially encoded subunits (Enriquez, 1995) . However, it has been reported recently, that in contrast to previous studies which was done in cybrid cell lines (Enriquez et al. 1995), the phenotypic expression of A8344G mutation in the tissues of affected individuals has been found to be a result of mechanism other than reduced accumulation of tRNA^{Lys} and/or reduced availabitlity of aminoacylated tRNA^{Lys}; it is a result of partial misacylation and/or incorrect interactions between the mutant tRNA^{Lys} and translation factors. This report was based on an assay that combines tRNA oxidation and circularization (Börner et al, 2000).

Mitochondrial Encephalomyopathy with Lactic Acidosis and Stroke-like Episodes (MELAS)/Mitochondrial Diabetes Mellitus (MDM). MELAS is probably the commonest of the mitochondrial encephalomyopathies. An A to G transition in the tRNA$^{Leu(UUR)}$ gene at nucleotide position 3243 (Table 1.4) is most frequently found in MELAS (Goto et al. 1990); this mutation alters the dihydrouridine loop in tRNA$^{Leu(UUR)}$. This mutation also inactivates the transcriptional terminator encompassed within the tRNA$^{Leu(UUR)}$ gene and downstream from the rRNA genes. Therefore, the MELAS mutation may reduce the efficiency of translation and later the ratio of mitochondrial rRNA and mRNA transcripts (Goto et al. 1990; Hess et al. 1991). Recently, it has been reported that using an assay that combines tRNA oxidation and circularization, the tRNA$^{Leu(UUR)}$ carrying the A3243G mutation shows decreased availability of aminoacylated tRNA$^{Leu(UUR)}$, indicative of a specific defect of the mutant molecule in stability and/or aminoacylation (Borner, 2000). A recent report on cell lines that are nearly homoplasmic for the mutation showed a strong (70-75%) reduction in the level of aminoacylated tRNA$^{Leu(UUR)}$ and a decrease in mitochondrial protein synthesis rate. However, there is no significant correlation between synthesis defect of the individual polypeptides and number or proportion of UUR codons in their mRNAs, indicating that another step other than elongation, may be affected. Further analysis showed several lines of evidence indicating the protein synthesis defect in A3243G MELAS mutation-carrying cells is mainly due to a reduced association of mRNA with ribosomes, possibly as a consequence of the $tRNA^{Leu(UUR)}$ aminoacylation defect (Chomyn et al.).

When the mutant mtDNA present in a high percentage (>85%) (Goto et al. 1990), MELAS is characterized by recurrent stroke-like episodes, migraine, nausea and vomiting, which usually present during childhood or adolescence (Pavlakis et al. 1984). Myopathy, cardiomyopathy, gastrointestinal dysfunction, deafness and non-insulin diabetes mellitus are also associated with this mutation (Ciafaloni et al. 1992). When present in a low percentage (5 to 30% of mtDNAs), this mutation is associated with maternally inherited diabetes mellitus (MDM) and deafness (van den Ouweland et al. 1992; Kadowaki et al. 1994; Gerbitz et al. 1995).

Aminoglycoside-induced Deafness/Non-syndromic Deafness. The A1555G mutation in the mitochondrial small (12S) rRNA gene was the first mtDNA mutation to be strongly associated with maternally inherited deafness trait: aminoglycoside-induced deafness, a cochlear damage due to hypersensitivity to normal doses of antibiotics (Fischel-Ghodsian et al. 1993; Hutchin et al. 1993; Prezant et al. 1993), and congenital and progressive deafness that is not the result of exposure to aminoglycoside

Table 1.4. MERRF and MELAS mutations.

MtDNA Mutation	Gene	Clinical Manifestation	References
G583A	$tRNA^{Phe}$	MELAS	(Hanna et al. 1998)
G1642A	$tRNA^{Val}$	MELAS	(de Coo et al. 1998)
A3243G	$tRNA^{Leu}$	MELAS, MDM	(Goto et al. 1990)
A3252G	$tRNA^{Leu}$	MELAS	(Morten et al. 1993)
C3256T	$tRNA^{Leu}$	MELAS	(Moraes et al. 1993)
T3271C	$tRNA^{Leu}$	MELAS	(Goto et al. 1991)
T3291C	$tRNA^{Leu}$	MELAS	(Goto et al. 1994)
A8296G*	$tRNA^{Lys}$	MERRF, MDM+Deafness	(Kameoka et al. 1998)
A8344G*	$tRNA^{Lys}$	MERRF	(Shoffner et al. 1990; Yoneda et al. 1990)
T8356C*	$tRNA^{Lys}$	MERRF	(Silvestri et al. 1992)
G8363A	$tRNA^{Lys}$	MELAS	(Ozawa et al. 1997)
T3308C	ND1	MELAS	(Campos et al. 1997)
T9957C	COXIII	MELAS	(Manfredi et al. 1995)
A11084G	ND4	MELAS	(Lertrit et al. 1992)
G13513A	ND5	MELAS	(Santorelli et al. 1997)

antibiotics, which is caused by cochlear damage uncomplicated by other morphological features (Prezant et al. 1993; Matthijs et al. 1996).

The A1555G mutation affects a highly conserved domain of the mitochondrial 12S rRNA, which has two single-stranded regions with conserved sequence, separated by two structurally conserved stem-loops in organisms as diverse as bacteria, plants, invertebrates and mammals (Noller 1984; Gutell et al. 1985; Neefs et al. 1991). In the case of aminoglycoside-induced deafness, the mutation causes mitochondrial ribosomes become the targets of aminoglycosides, similar to that seen in the evolutionary related bacterial ribosomes (Prezant et al. 1993). However, this hypothesis could not be applied to congenital and/or progressive deafness patients that have never been exposed to aminoglycoside antibiotics. Several hypothesis regarding this matter has been proposed; first, there is/are nuclear factor(s) involvement that modulate the diversity in biochemical phenotype between symptomatic and asymptomatic individuals (Jaber et al. 1992; Guan et al. 1996; Guan et al. 2000), and second, the A1555G mutation causes a general reduction in translational efficiency that manifested as diminished ability to repair cochlear damage from a variety of sources, including noise (Usami et al. 1997) .

MtDNA Re-arrangement Mutations

Diseases resulting from mtDNA re-arrangements have been associated with four major clinical phenotypes: the ocular myopathies, including the Kearns-Sayre syndrome (KSS) and chronic progressive external ophthalmoplegia (CPEO) (Holt et al. 1988; Moraes et al. 1989; Poulton 1993), Pearson marrow/pancreas syndrome (Rotig et al. 1989; Rotig et al. 1990) adult onset diabetes mellitus and deafness (Ballinger et al. 1992), and late-onset myopathy (Manfredi et al. 1997). Two important features of the mtDNA deletions in relation to pathogenesis are: (a) most of the patients with partially deleted mtDNA reported so far occur sporadically, with only rare exceptions, and (b) the deleted mtDNA is always found in tissues in a heteroplasmic manner. These characteristics suggest that mostly the mtDNA deletions have occurred *de novo*; either very early during embryonic development or at a relatively late, post-developmental stage (Marzuki et al. 1997) .

More than 100 mtDNA re-arrangements have been identified in mitochondrial degenerative diseases (for review see Wallace et al. 1995) Two of the first mtDNA re-arrangements to be detected were large deletions (modal length 4,977 bp, Holt et al. 1988) and partial duplications (Poulton et al. 1995; Rotig et al. 1992) of the genome. Deletions are recognized more frequently than duplications. Most of the deleted area in the genome are flanked by direct repeats (Schon et al. 1989). The most common deletion found in 30-50% of patients, is flanked by 13-np repeats

(5'-ACCTCCCTCACCA) between nps 8468 and 13446, and removes 4977 bp of the sequence (Holt et al. 1988; Schon et al. 1989; Shoffner et al 1989). Several mechanisms have been proposed for the origin of the deletions, one favoured mechanism postulates that deletions arise during asymmetric mtDNA replication as a consequence of slipped replication and template switching (Shoffner et al. 1989). Deletions in the mtDNA are highly heterogenous in both size and position. However, in most cases, the deletions always spare the two origins of replication, the H-strand origin and the L-strand origin, respectively, which located on opposite sides of the genome. It is presumed that deletions that eliminate origins retard replication and do not accumulate in somatic tissues. An exception, a 10.4 kb deletion which spanned the L-strand origin (O_L), was reported by Ballinger et al (1992). Normally, replication starts at the H-strand origin (O_H) and proceeds two-thirds of the way around the genome to the O_L before synthesis of the complementary strand can start. The survival of the patients with the deleted O_L could be explained by the finding of duplications and deletions dimers (but not deletion monomers) in the genome (Ballinger et al. 1994), presumably the dimers are able to replicate because they have two copies of O_H.

MtDNA Mutations and Biochemical Defects in Tissue Culture Model and Transmitochondrial Cell Lines

The phenotypic expression of a mitochondria disorder as a consequence of mtDNA mutation in an organism as complex as mammals, is regulated by many complicated factors, including cellular and hormonal interactions, as well as environmental factors. In such a complex system, it would be complicated to study and elucidate the pathomechanism of a disease. In more than two decades, fibroblast cell line has been utilized as a model to study in vitro ageing.

Human cell culture has been used and manipulated to a large extent to study the biochemical defects related to mtDNA and nuclear DNA mutations associated with respiratory enzyme deficiencies and/or mitochondrial diseases. Studies on mitochondrial diseases in fibroblast, myoblast and lymphoblastoid cell lines have been conducted.

More than 10 years ago (Gregoire et al. 1984; Desjardins et al. 1985; 1986) by long term exposure of avian fibroblast cells to low concentration of the chelating agent ethidium bromide, a cell line devoid of mtDNA has been established. These cells have become pyrimidine auxotroph because of the deficiency of the respiratory chain-dependent dihydroorotate dehydrogenase (Gregoire et al. 1984; Desjardins et al. 1985). A few years later using a similar method, King and Attardi (1989) have been successful in generating human ρ^0 cells, which have been completely depleted of mtDNA and are respiratory deficient, thus rely exclusively on glycolysis for their energy requirements, as well as becoming pyrimidine auxotroph. These ρ^0 cells were then fused with various mitochondria donor exhibited a respiratory phenotype that was distinct from that of the ρ^0 recipient cells. The resulting transformant cells are called cybrids, and the fusion was able to restore the cells to be respiratory competent. This technology has opened a way, which enabled studies of the analysis of the functional capacity of mtDNA from normal individuals as well as from individuals carrying mtDNA and/or nuclear DNA mutations associated with mitochondrial disorders (for examples see Chomyn et al. 1994; King and Attardi 1989; Bodnar et al. 1993; Trounce et al. 1994; Dunbar et al. 1995; Marusich et al. 1997; Raha et al. 1999; Tang et al. 2000).

Mitochondrial Dysfunction and Transgenic Mice

The use of human and cybrid cell lines have been proven to be very useful in studying the pathogenicities of mtDNA and/or nuclear DNA mutations, which have been identified in association with the human ageing and age-associated disorders including diabetes mellitus and neurodegenerative diseases (Wallace et al. 1995). Nevertheless, there is still no convincing *in vivo* evidence that the accumulation of these pathogenic mutant mtDNAs in tissues is the sole cause of various clinical phenotypes. Establishment of animal models with a wide spectrum of mtDNA mutations may provide a model system to study the role, transmission, and distribution of the mtDNA mutations in tissues.

It was reported that transgenic mice, which demonstrate tissue-specific rapid accumulation of mutations in the mtDNA, have been constructed (Zhang et al. 2000). The transgenic mice carry a proofreading deficient mouse DNA pol γ, which is known to harbour two activities, that of polymerase and 3'→5' exonuclease activities, which were contained within the same polypeptide. The 3'→5' exonuclease activity functions in proofreading of newly replicated DNA, and activation if this exonuclease will reduce the fidelity of the mtDNA replication (Kunkel and Mosbaugh 1989).

Another report (Inoue et al. 2000) mentioned that about a 4.7 kb ΔmtDNA has been introduced into mouse zygotes by electrofusion of pronucleus-stage embryos with several enucleated cytoplasts of cybrid cells containing the deleted mtDNA. The existence of deleted, partial duplicated, and wild type mtDNAs were observed. Mitochondrial dysfunction(s) were detected (negative COX activity determined by histochemical staining were detected in fibres possessing more than 85% of the mutant mtDNA). The deleted mtDNA, surprisingly, were transmitted from mother to offspring.

The transgenic mice may prove to be a valuable resource for studying the mechanisms of mtDNA mutations generation, transmission and pathogenic expression on mitochondrial and cellular function.

Nuclear Involvement in Mitochondrial Disorders

As mentioned previously in this chapter, the mitochondria respiratory chain produces most of the cellular energy, and the enzyme complexes responsible for this energy metabolism, located in the mitochondrial inner membrane, are formed as an assembled polypeptide synthesized in both cytoplasmic and mitochondria. Only a few of the components of the respiratory enzyme complexes are encoded by mtDNA, as well as other proteins essential for the maintenance of mitochondria structure and function. Most of the components of the mitochondrion's own genetic system are encoded by nuclear genes.

Mitochondrial diseases resulting from nuclear-encoded defects have been increasingly recognized. The mitochondrial diseases associated with nuclear-encoded defects are categorized as: (1) diseases associated with nucleo-cytoplasmic interactions; (2) diseases caused by mutations in the nuclear-encoded OXPHOS subunits; and (3) diseases related to mutations in the nuclear gene encoding non-OXPHOS mitochondrial protein, but the defects indirectly inactivating OXPHOS or destabilizing the mtDNA; (4) diseases caused by mutations in the nuclear gene encoding non-mitochondrial protein (see Schapira 1999; Beal 2000; Leonard and Schapira 2000, for recent reviews).

Diseases Associated with Nuclear-Mitochondrial DNA Interactions.

Copy Number Mutations. A new class of mitochondrial disease has been reported which does not involve qualitative errors in mtDNA, but quantitative depletion of the mtDNA in clinically and biochemically affected tissues (Moraes et al. 1991). This disorder was identified in a group of infants, suggesting that it may be an important cause of mitochondrial dysfunction in neonates and infants. The level of mtDNA depletion in the affected tissues varies, and can be up to 98%, while unaffected tissues have relatively normal levels of mtDNA (Moraes et al. 1991; Poulton et al. 1995; Bodnar et al. 1993; Mariotti et al. 1995).

The depletion in mtDNA levels has been associated with the reductions in mtDNA gene products, but not nuclear OXPHOS gene products, which demonstrate the association between the copy number defect and the biochemical defect (Moraes et al. 1991). It has been suggested that the mtDNA replication is arrested due to *trans*-acting nuclear-encoded factor(s) involvement in mtDNA replication process (Rahman et al. 1999; Moraes et al. 1991; Bodnar et al. 1993), since regulation of mtDNA copy number is closely related to the regulation of mtDNA replication (for review see Taanman 1999). The tissue specific and neonatal expression of symptoms suggest that the factor might be tissue specifically and developmentally regulated, and is most likely only to be expressed after a certain number of cell divisions.

Autosomal Progressive External Ophthalmoplegia/Mitochondrial Myopathy. There are two types of the autosomal mitochondrial myopathy, one is autosomal dominant (autosomal dominant progressive external ophthalmoplegia/ad-PEO) (Haltia et al. 1992), the other is autosomal recessive (mitochondrial neurogastrointestinal encephalomyopathy/ MNGIE) (Carrozzo et al. 1998; Hirano et al. 1994).

The ad-PEO is an adult-onset mitochondrial disorder with large-scale mtDNA deletions; clinically characterized by muscle weakness, most severely in the ocular muscles (progressive external ophthalmoplegia, ptosis), exercise intolerance, and sometimes with ataxia, depression, hypogonadism, hearing deficit, peripheral neuropathy, and cataract (Suomalainen et al. 1995). The percentage of ragged-red and cytochrome c oxidase-negative fibers are quite high in patients with ad-PEO (Carrozzo et al. 1998). The autosomal dominant inheritance of the disease, instead of maternal inheritance like any other mitochondrial diseases, pointed to nuclear gene defect, which predisposes the accumulation of *de novo* mtDNA deletions. Patients with ad-PEO harbored approx. 31% of deleted mtDNA species in muscle (Carrozzo et al. 1998). Three distinct autosomal loci for this disorder have been identified, on chromosomes 10q23.3-24.3

(Suomalainen et al. 1995), 3p14.1-21.2 (Kaukonen et al. 1996), and 4q34-35 (Kaukonen et al. 1999), respectively. Located at the critical region of chromosome 4q34-35 is the gene encoding the heart- and skeletal muscle-specific isoform of the adenine nucleotide translocator (ANT1). Human ANT exists as three isoforms: the ANT1, expressed predominantly in terminally differentiated tissues like skeletal muscle, heart and brain, the ANT2, expressed mainly in proliferating and regenerating tissues, and the ANT3, expressed ubiquitously (Stepien et al. 1992). ANT exchanges mitochondrial matrix ATP for cytosolic ADP across the mitochondrial inner membrane. In addition to the translocase activity, ANT is a core structural element of the mitochondrial permeability transition pore (MPTP) (Brustovetsky and Klingenberg 1996).

While adPEO patients are associated with myopathic features, the MNGIE patients are associated with multisystem disorders, including severe gastrointestinal dysmotility, cachexia, ptosis and/or ophthalmoparesis, peripheral neuropathy, and leukoencephalopathy. The percentage of ragged-red and cytochrome c oxidase-negative fibers are much lower compared to the ad-PEO patients, as well as the proportion of deleted mtDNA in muscle (approx. 9.7%, respectively) (Carrozzo et al. 1998). The disease is caused by mutations in the thymidine phosphorylase (*TP*) gene (Nishino et al. 1999; Nishino et al. 2000); mapped to chromosome 22q13.32-qter (Hirano et al. 1998). It has been hypothesized that inactivation of TP alters cellular thymidine (nucleoside and nucleotide) pools, leading to impaired replication, repair, or maintenance of mtDNA, or a combination of all three (Nishino et al. 1999).

Mutations of Nuclear Genes Encoding OXPHOS Protein

Several mitochondrial diseases resulting from mutations in nuclear-encoded OXPHOS genes have been identified. These diseases, although exhibit Mendelian inheritance patterns, yet share many of the clinical features of mtDNA mutations. Mutations in a number of nuclear genes have been associated with Leigh's or Leigh-like syndrome with pyruvate dehydrogenase, mitochondrial complexes I, II, and IV deficiencies (for review see Dahl 1998), including: (1) mutations in the gene encoding the E1α subunit of pyruvate dehydrogenase complex (PDHC) (Matthews et al. 1993); (2) the NDUFS8 subunit of mitochondrial complex I (possible binding site for iron-sulfur cluster N-2) (Loeffen et al. 1998); (3) the NDUFS7 (PSST) of mitochondrial complex I (Triepels et al. 1999), the flavoprotein of mitochondrial complex II (Bourgeron et al. 1995), and the SURF-1 protein associated with complex IV assembly and maintenance (Zhu et al. 1998; Tiranti et al. 1999). Other nuclear gene encoding OXPHOS protein mutations that have been identified were: (1) a 5-bp duplication in the gene encoding for the NDUFS4 (AQDQ) subunit of complex I, manifested as encephalomyopathy (van den Heuvel et al. 1998); (2) mutations in the NDUFV1 subunit of complex I associated with leukodystrophy and myoclonic epilepsy (Schuelke et al. 1999).

Diseases Caused by Mutations of Nuclear Genes Encoding Non-OXPHOS Mitochondrial Protein

OXPHOS dysfunction that linked to defects of non-OXPHOS mitochondrial proteins is predominantly clinically expressed as neurodegenerative diseases, due to indirectly inactivation of OXPHOS or destabilization of the mtDNA. Friedreich's ataxia is one of the diseases that falls into this category, it is an autosomal recessive neurodegenerative disease characterized by cerebellar ataxia, peripheral neuropathy, skeletal abnormalities, hypertrophic cardiomyopathy, diabetes mellitus (see Schapira 2000). The causative mutation is mostly an abnormally expanded GAA triplet repeat within the first intron of the gene encoding frataxin on chromosome 9q13, which is targeted to the mitochondrial inner membrane (Patel et al. 2001). There is an increased iron deposition in the heart in Friedreich's ataxia patients, suggesting that frataxin may be involved in intramitochondrial iron handling (see Puccio et al. 2000) for recent review]. Biochemical analysis of tissues from patients demonstrate severe deficiencies of mitochondrial complexes I, II, and III and of the Krebs cycle enzyme aconitase, all of which contain Fe-S clusters, which are known to represent critical targets for oxygen free radicals. The iron overload in the matrix of mitochondria would cause stimulation of H_2O_2 conversion to OH^- via the iron-catalyzed Fenton reaction, which inactivates the mitochondrial Fe-S center enzyme complexes (see Puccio et al. 2000).

Another autosomal recessive neurodegenerative disorder due to mutations in a mitochondrial protein is hereditary spastic paraplegia, which is associated to mutations in paraplegin, a nuclear-encoded mitochondrial metalloprotease (protease-like adenosine triphosphatase/ATPase) (Casari and Rugarli 2001). This disorder may present in childhood or early to mid adulthood with gradual onset and progression of weakness and spasticity (see Fink 2001).

Wilson's disease is also another autosomal recessive neurological disorder which is caused by mutation in nuclear gene encoding mitochondrial protein, a copper-transporting P-type ATPase, the WND protein. This disorder is characterized by abnormalities in copper homeostasis, which caused copper accumulation in liver and basal ganglia, and resulted in a spectrum of hepatic and neurological abnormalities (Buiakova et al. 1999; Hamza et al. 1999). Several studies have shown increased oxidative damage in the liver and peripheral tissues of individuals with this disease (Nair 1999).

Diseases Associated with Mutations in the Nuclear Gene Encoding Non-Mitochondrial Protein

Huntington's disease is caused by an abnormally expanded CAG repeat within the huntingtin gene on chromosome 4 (see Grunewald and Beal

1999). The function of huntingtin is still not known. Biochemical studies of HD in postmortem tissues show clear evidence for OXPHOS defects, with severe deficiencies in mitochondrial complexes II and III, and a smaller decrease in complex IV activity (Gu et al. 1996; Browne et al. 1997; Tabrizi et al. 1999), eventhough huntingtin is not thought to be a mitochondrial protein. Therefore, the cause of the mitochondrial changes is unexplained.

Several line of evidence showed loss of electron transport chain activity in multiple tissues from individual with Parkinson's disease.(PD) (see Schapira et al. 1992; Shults et al. 1999; Greenamyre et al. 1999). The dopaminergic nerve cell death in the substantia nigra underlies the major clinical features of PD, although the cause is still not known (see Schapira 1997). It has been suggested that defects of mtDNA may be responsible for the mitochondrial abnormality in PD (Gu et al. 1997).

Complex IV deficiency has been described in Alzheimer's disease brain (AD) (Bonilla et al. 1999). Immunostaining for cytochrome oxidase subunits II and IV showed a reduction in Purkinje cells in individuals with AD; the reduction is much more marked for the mtDNA-encoded subunit II than that of the nuclear DNA encoded subunit IV (Ojaimi et al. 1999). However, recently a study was unable to demonstrate electron transport chain deficiency in cybrid cell lines constructed from a synaptosomal fraction of autopsied brain tissue or platelets of AD individuals (Ito et al. 1999).

CHAPTER 2

MICROPHOTOMETRIC ANALYSIS OF NADH-TETRAZOLIUM REDUCTASE DEFICIENCY IN FIBROBLASTS OF PATIENTS WITH LEBER'S HEREDITARY OPTIC NEUROPATHY

INTRODUCTION

Mutations in the mitochondrial DNA (mtDNA) have been shown to be the underlying molecular defects in a number of adult onset neuromuscular and infantile multi-systems disorders. Many of these mutations have been now identified, but the molecular mechanisms responsible for the disease process have not been in general elucidated. A typical example is Leber's hereditary optic neuropathy (LHON), which is an inherited degenerative disorder characterized by visual failure resulting from a bilateral optic atrophy, with multiple organ involvement in certain families. Around 50 - 70% of LHON pedigrees carry a mutation at nt G11778A in the ND4 gene of the mtDNA, assumed to affect the function of this subunit of the respiratory complex I (see Riordan-Eva and Harding 1995; Wallace 1999). The relationship between this mutation, the resulting biochemical defects and its clinical manifestation, however, is still not well understood and appears to be complex.

Enzymatic biochemical disorders consequential of a mtDNA mutation and their clinical manifestations appear to be the outcome of an interplay between the causal mutation and other sequence variants of the mtDNA, and between the nuclear and the mitochondrial genetic systems (see Hanna and Nelson 1999, for recent reviews; Wallace 1999; Zeviani and Antozzi 1997). The expression of pathological mtDNA mutations is determined and modulated also by apparently random cellular events during development. MtDNA carrying a pathological mutation often co-exists in cells with the normal mtDNA in a heteroplasmic manner, and the two populations of mtDNA segregate randomly during cell division resulting in varying degree of heteroplasmicity in different cells. The age-related somatic accumulation of mtDNA mutations proposed to be an important factor in the aging process (Linnane et al. 1989; Trounce et al. 1989) is also a random cellular event. The adult onset manifestation of most pathological mitochondrial mtDNA mutations has been suggested to be the result of the expression of these mutations against a background of an age-related decline in the tissue capacity for oxidative metabolism. Thus, the respiratory enzyme deficiencies observed in tissues as the consequence of pathological mtDNA mutations (see Collins et al. 1991; and Keightley et al. 1996, for examples), and that observed in aging tissues (see Muller Hocker 1990), are in general of a mosaic nature. Important information regarding the phenotypic expression of mtDNA mutations, therefore, could be gained from the ability to analyze biochemical and molecular defects at the single cell level.

Histochemical staining is commonly used to asses respiratory enzyme deficiency associated with mtDNA mutations in various neuromuscular disorders at the single cell and tissue levels (Collins et al. 1991; Dubowitz and Brooke 1973), but it has not been possible in the past to obtain quantitative data from such studies. The introduction of microphotometric facilities in recent years, however, has provided a means for the measurement of cellular enzyme activities microscopically. In the study presented in this chapter, I have assessed the utility of the microphotometric approach to the single cell analysis of the respiratory enzyme deficiency, and employed this approach to examine whether the expected functional defect in the mitochondrial respiratory complex I can be detected in LHON.

MATERIALS AND METHODS

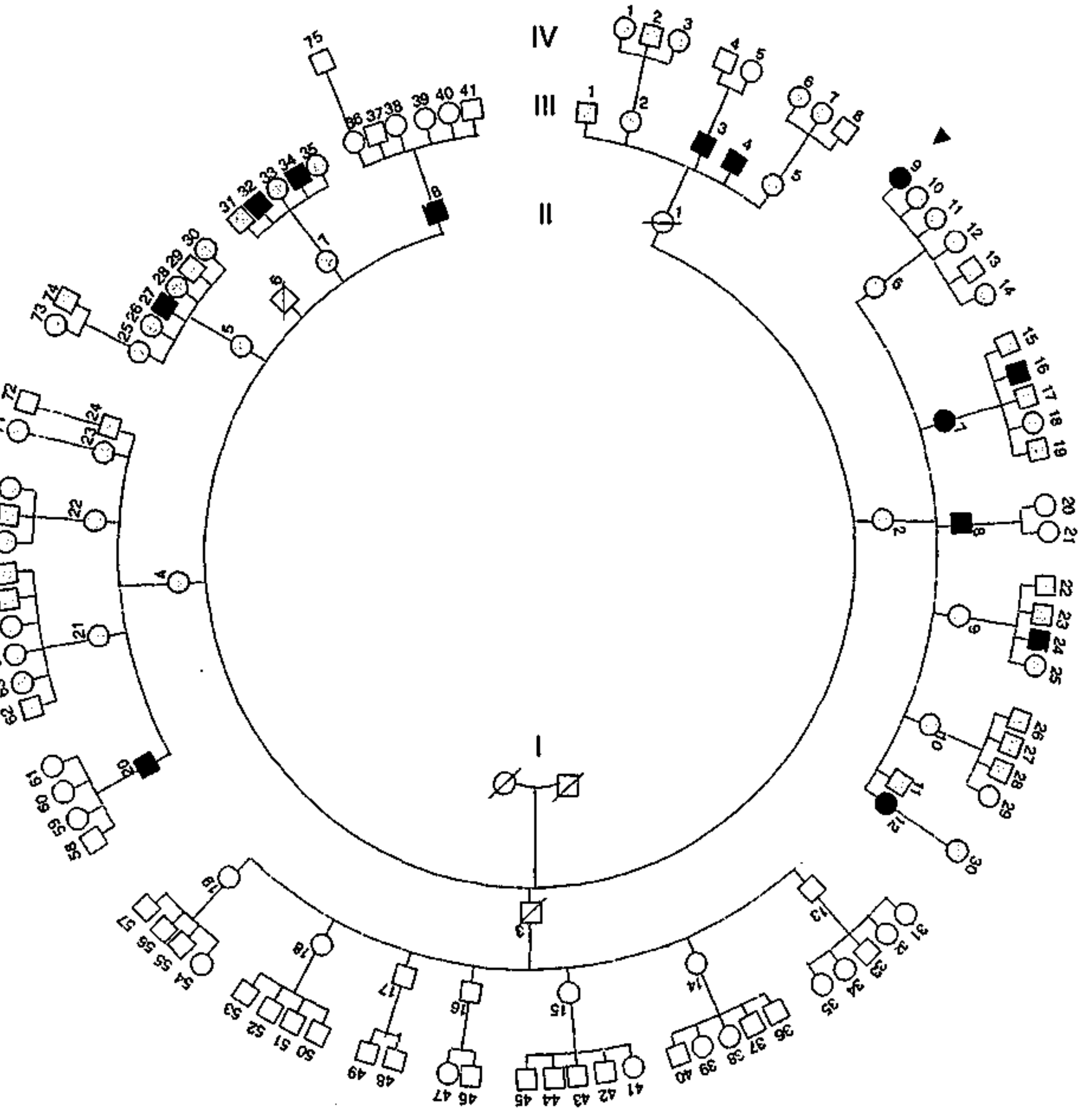
Patients and Fibroblast Culture

The LHON patients included in the present study are members of a large Chinese family from Jambi in the island of Sumatera, Indonesia, recently reported (Sudoyo et al. 1998) (Fig. 2.1). Skin biopsies were taken from two members of this family with informed consent. Both family members carry the G11778A causal mutation and are from the third generation, EIJbIII-1-94 (46 yr) who did not show any clinical expression of LHON and EIJbIII-4-94 (33 yr) who exhibited the clinical signs of LHON. Skin biopsies were obtained also from two normal individuals of similar age (EIN-2-95 and EIN-3-95) who are not related to the LHON family, and are also of Chinese ethnic background.

Biopsies were taken from the ventral side of the arm (3.5 mm) under local anaesthetic with lidocaine. Biopsy materials were washed with Minimal Essential Medium (MEM; Gibco-BRL) supplemented with 15% fetal bovine serum (FBS), 100 IU penicillin-100 µg/ml streptomycin and 5 µg/ml Fungizone (Rooney and Czepulkowski 1987; Sly and Grubb 1979), and also with 100 µg/ml sodium pyruvate and 50 µg/ml uridine (King and Attardi 1989). The biopsied skins were diced and cultured in the same medium under cover slips for 4-5 weeks. The fibroblast cells were then

Figure 2.1. A LHON Pedigree from Jambi, Sumatra, carrying the G11778A LHON mutation.

A four generation pedigree showing the following members of the family: □▩■ = male, ○◉● = female, ■● = affected, ▩◉ = asymptomatic but carry the 11778G>A mtDNA mutation, □○ = asymptomatic no 11778G>A mutation, □○ = deceased, ▲ = proband.


IV
III
II
I

propagated further or prepared for storage by freezing (see Sly and Grubb 1979).

Detection of the mtDNA G11778A LHON Mutation

Total DNA samples were prepared from fibroblasts as described elsewhere (Lertrit et al. 1992), and subjected to PCR amplification using a primer pair which amplifies a 214 bp fragment of the mtDNA between nt 11728 - nt 11942 (Sudoyo et al. 1998). The reverse primer has been modified to introduce a *BclI* site in combination with the G11778A mutation. The amplified fragment was then digested with *BclI* restriction endonuclease (Gibco BRL) to detect the presence of the LHON mutation.

NADH-tetrazolium Reductase Activity and Microspectrophotometry

The respiratory complex I activity was determined by measuring the NADH-tetrazolium reductase activity histochemically, essentially as described by Dubowitz and Brooke (1973). For this purpose, fibroblasts were grown first on the surface of a glass cover slip to near confluence. The reaction mixture consists of 0.2 M phosphate buffer pH 7.4 containing 0.1 M sodium lactate, 0.1% lactate dehydrogenase (LDH; Boehringer Mannheim), 0.5 mg/ml NAD (Boehringer Mannheim), and 0.5 mg/ml nitro blue tetrazolium (NBT; Boehringer Mannheim). Cells were incubated in the reaction mixture at 37°C, in a dark moist chamber, for the time specified (between 0-60 min). The enzyme reaction was terminated by washing twice with 0.05 M phosphate buffer at the room temperature. NADH-tetrazolium reductase converts the soluble colourless form of oxidized NBT into an insoluble blue reduced product, the intensity of which was evaluated microphotometrically using a microscope photometer MSP21 (Carl Zeiss, Germany). The spectrometric measurement was carried out at 510-525 nm, employing a 0.25 mm pinhole, at various areas of the cells as indicated in Results and Discussion. The measured field diameter (µm), calculated using the formula

$$\frac{1000 \times \text{diaphragm diameter (mm)}}{V_{\text{objective}} \times \text{Optovar-factor}} \quad \text{was } 6.25\ \mu\text{m}.$$

Measurement of Arrhenius Kinetics

The NADH-tetrazolium reductase activity was determined histochemically as described above at temperatures ranging from 9 to 40°C (Marzuki et al. 1975; Orian et al. 1984). Briefly, cells were incubated in a dark moist chamber in the reaction mixture at 9, 12 and 15°C for 1 to 5 h with an interval of 1 h; at 20, 25, 27 and 30°C for 30 to 120 min with an interval of 30 min; and at 33, 35, 36, 38 and 40°C for 15 to 60 min with an interval of 15 min. The reaction was terminated by washing twice with 0.05 M phosphate buffer at room temperature. The NADH-TR activities assayed at various temperatures described above were shown to be linear for at least 5 h for incubation temperatures of 9, 12 and 15°C, 120 min for incubation temperatures of 20, 25, 27 and 30°C, and 60 min for incubation temperatures of 33, 35, 36, 38 and 40°C. The intensity of the insoluble blue reduced product was quantitated microphotometrically at 510-525 nm employing a 0.25 mm pinhole as described previously. The rate was calculated from the linear part of the curve and expressed as $\Delta A_{510\text{-}525}$ unit/10 min.

RESULTS

Quantitative Assessment of the NADH-Tetrazolium Reductase Activity

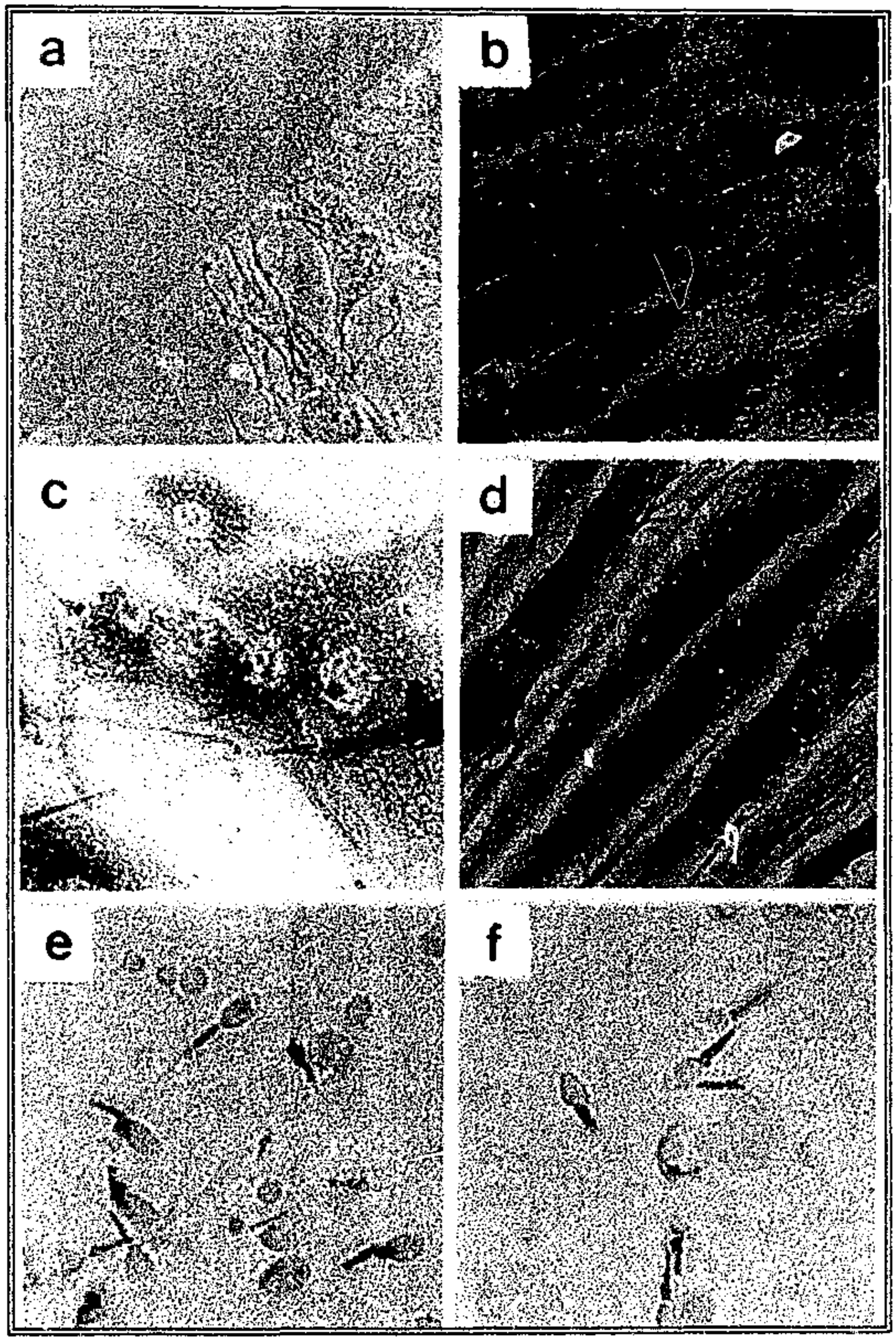
The G11778A mutation is associated with an arginine to histidine amino acid change in the conserved residue 340 of the ND4 subunit of the respiratory complex I and thus expected to affect primarily the activity of this enzyme complex. I have thus focused the present study on the respiratory enzyme complex I, and employed a well-characterized NADH-tetrazolium reductase assay system (Collins et al. 1991; Dubowitz and Brooke 1973) to measure the partial reaction of the respiratory complex I in a histochemical setting for a microphotometric application. This histochemical staining is routinely used in the qualitative assessment of respiratory complex I deficiency in neuromuscular disorders associated with mtDNA mutations, and measures the complex I-catalysed transfer of electron between NADH and an artificial electron acceptor, nitro blue tetrazolium (NBT). In the design of the assay procedure, steps have been introduced in the histochemical preparation to ensure the permeability of the plasma and mitochondrial membranes to the various components of the reaction mixtures. It relies on an external regenerating system to maintain an excess level of the NADH substrate throughout the length of the reaction. The assay, therefore, directly measures the activity of the respiratory complex I as the rate limiting step in the complex reaction system (Dubowitz and Brooke 1973).

The NADH tetrazolium reductase (NADH-TR) activity of the respiratory complex I was first examined in the normal control fibroblast cell lines. Intense blue granules were observed within the fibroblasts after 60 min of incubation in the reaction mixture (Fig. 2.2b). This staining is fully dependent on the supply of NADH, as in the absence of the NADH regenerating system the colour observed was similar to that at the beginning of incubation (Fig. 2.2a).

The quantitative measurement of the fibroblasts NADH-TR activity was complicated by the observation of an apparently non-homogeneous distribution of the activity within the cells. A definitive pattern of distribution of the NADH-TR activity was noted within the fibroblast cells, with the highest activity observed around the nucleus, gradually decreasing toward the periphery (Fig. 2.2b).

Figure 2.2. Measurement of the NADH-Tetrazolium Reductase Activity.

Fibroblasts isolated from a normal individual (EIN-2-95) were grown on a cover slips to near confluence, and stained for the NADH-tetrazolium reductase activity as described in Materials and Methods. Shown are the stainings for: (a) NADH-tetrazolium reductase for 60 min, but omitting sodium lactate and LDH from the incubation mixture as an NADH regenerating system (top: light microscopy, bottom: phase contrast), and (b) NADH-tetrazolium reductase after 60 min of reaction; a similar picture to (a) was observed for the complete reaction at time 0. The fibroblasts were also stained for the succinate dehydrogenase activity (Dubowitz and Brooke 1973) for 90 min (c), and immunohistochemically for the respiratory complex I (essentially as described by Collins et al. 1991; Noer et al. 1992) with an antipeptide antibody raised as described by (Noer et al. 1992) against the carboxy terminal of NDUFS1, a nuclearly coded 75 kDa subunit of the respiratory complex I (Chow et al. 1991); peptide sequence (K)EGAQAVEEPSI], as shown in (d). As comparisons, sperm cells, in which the mitochondria are located exclusively in the midpiece, were stained for (e) NADH-tetrazolium reductase and (f) cytochrome *c* oxidase (Collins et al. 1991).


a
b
c
d
e
f

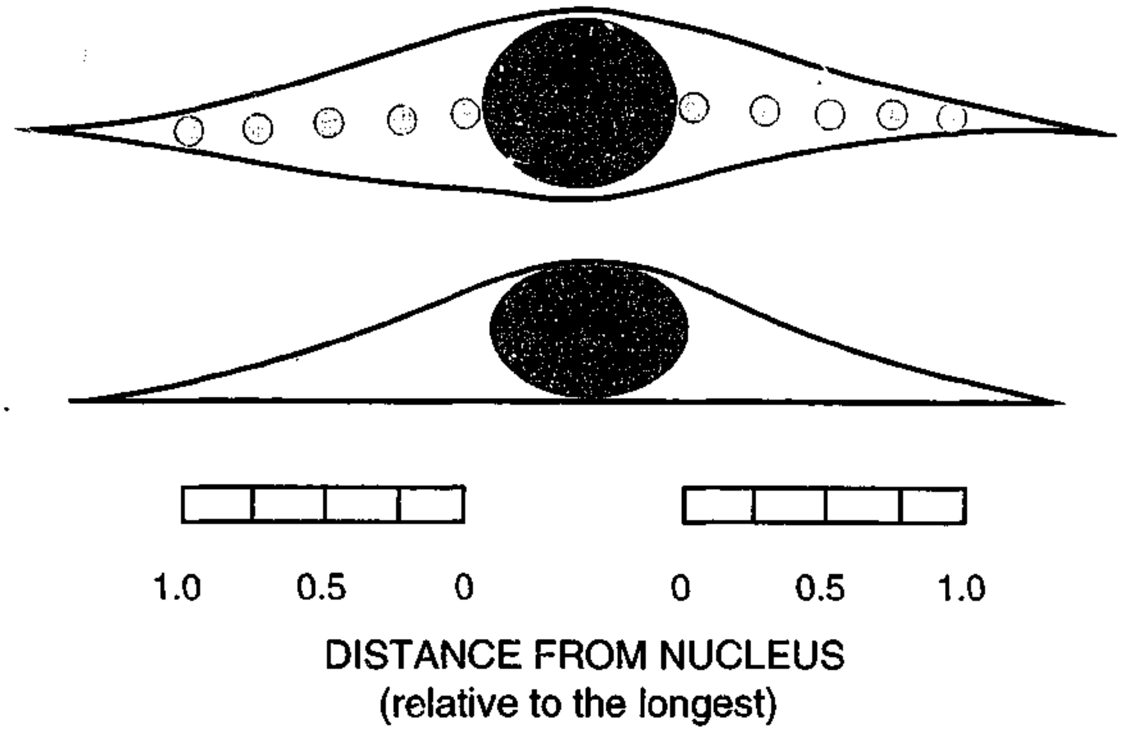
Electron microscopic examination of sectioned cells of the fibroblast, however, showed a homogeneous distribution of the mitochondrial structure within the cell (data not shown). It has been proposed, therefore, that the observed pattern of cellular distribution of the NADH-TR activity is the manifestation of the contour of the unsectioned fibroblast cells in the monolayer culture (Fig. 2.3 top). Thus, near the nucleus where the height of the fibroblast cells is at its maximum, an apparently high NADH-TR activity was observed because of the longer light path taken during the microphotometric measurement, whereas near the periphery of the cells the apparent activity is lowest.

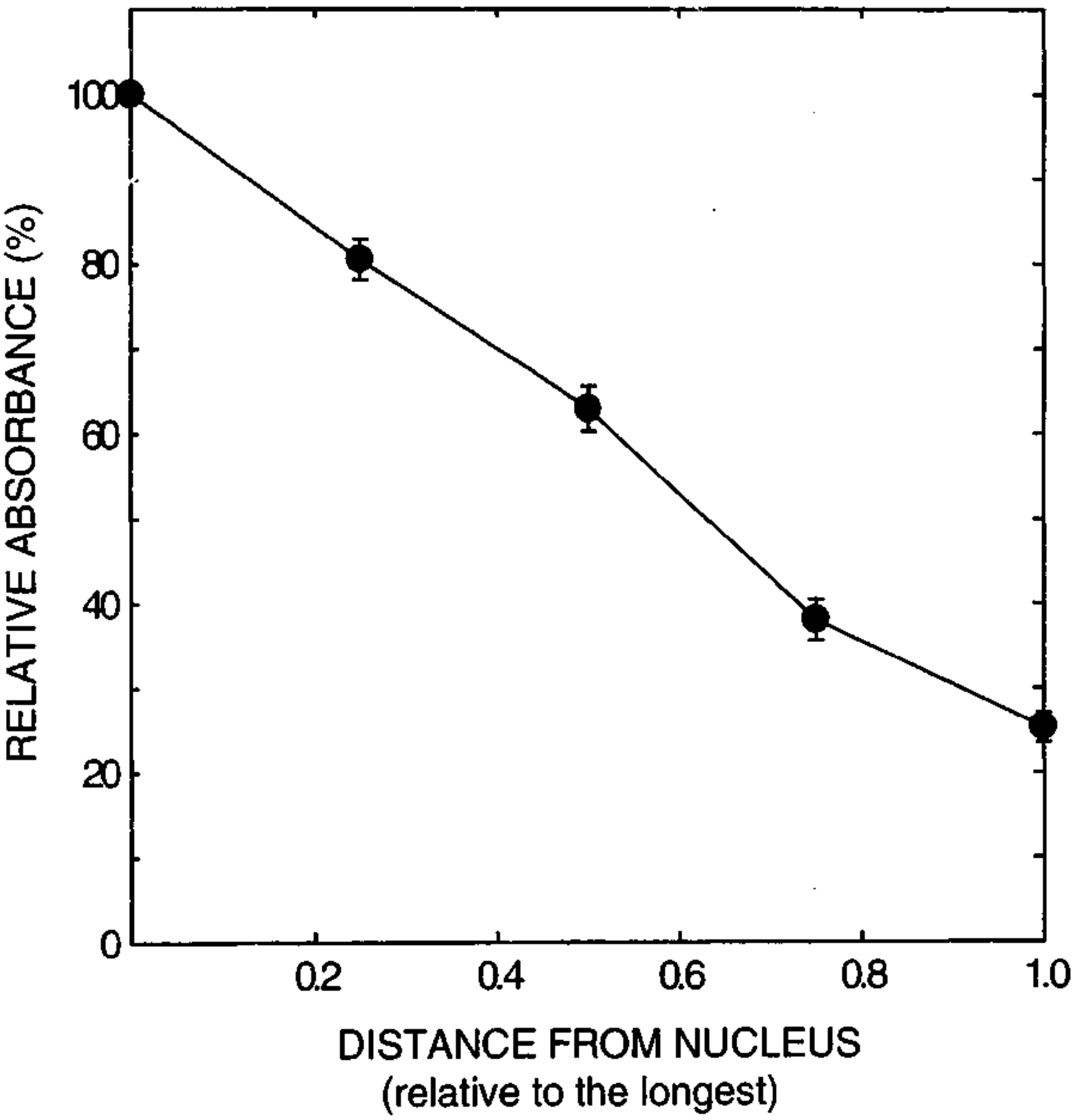
Another potential complication is the fact that although the NADH–TR activity is widely used to indicate the mitochondrial respiratory complex I activity in muscle biopsies from patients with neuromuscular disorders, this activity is present also in the endoplasmic reticulum, notably that associated with the NADH–cytrochrome b_5 reductase (Dubowitz and Brooke 1973). However, while the activity measured in this study might not be entirely due to the mitochondrial complex I activity, several observations indicate that the contribution of the microsomal NADH–TR activity is relatively small. Thus, it was observed that in sperm cells, where mitochondria are located distinctly and exclusively in the midpiece, the NADH–TR activity, and that of the cytochrome *c* oxidase, are associated only with the midpiece (Fig. 2.2e,f), with very little activity in the sperm head. Further, the intracellular granular distribution of the NADH–TR activity observed in the fibroblast cells (Fig. 2.2b) is very similar to that of the more specific succinate dehydrogenase (Fig. 2.2c) or the cytochrome *c* oxidase (data not shown). Finally, immunohistochemical staining (Collins et al. 1991; Noer et al. 1992) of the fibroblasts with an antipeptide antibody (Noer et al. 1992) to the carboxy terminal of the NDUFS1, a 75 KDa nuclearly coded subunit of the respiratory complex I (Chow et al. 1991), shows a similar pattern of mitochondrial distribution (Fig. 2.2d).

In the measurement of the NADH-TR activity, the following assumptions have been used: (a) the size of the nucleus of the subconfluent and thus slowly dividing fibroblasts is nearly homogeneous; (b) accordingly, most of the cells would have an equal height near the nucleus; and (c) it follows that at any distance relative to the peripheral membrane of the cells most of the fibroblasts would also have equal heights. The results obtained indeed support the above assumptions. First, when the NADH-TR activity was measured in the area adjacent to the nucleus (Fig. 2.4), the variation observed in the 100 cells examined was relatively small as indicated by the relatively low standard deviation (Mean±SD 0.44±0.11 $A_{510\text{-}525}$ unit/60 min and 0.53±0.12 $A_{510\text{-}525}$ unit/60 min for the normal EIN-2-95 and EIN-3-95 fibroblast cell lines, respectively). A near linear decrease was observed in the apparent activity when it was measured at various distances from the nucleus (Fig. 2.3), which presumably correlates with the shape of the fibroblasts. In line with the assumptions, the activity at a distance of 0.5 of the longitudinal length of the fibroblast also showed relatively small variation in 60 cells (0.30±0.13 $A_{510\text{-}525}$ unit/60 min for the normal EIN-2-95 fibroblast cell).

Figure 2.3. Microphotometric Quantitation of the NADH-Tetrazolium Reductase Activity.

The NADH tetrazolium reductase activity was measured microphotometrically at ten different sequential points using 0.25 mm pinhole along the fibroblast cells as indicated (O) and plotted as the intensity of the staining against the relative distance from the nucleus to the periphery (top). It is assumed that near the nucleus the height of the fibroblast cells is at its maximum, whereas near the periphery it is lowest (middle). Data presented are the Mean±SE of measurements from 100 cells (below).


1.0
0.5
0
0
0.5
1.0
DISTANCE FROM NUCLEUS
(relative to the longest)


RELATIVE ABSORBANCE (%)
100
80
60
40
20
0
0.2
0.4
0.6
0.8
1.0
DISTANCE FROM NUCLEUS
(relative to the longest)

It is important also to demonstrate for the purpose of the comparison of the activity in the various fibroblast cell lines that the measurements were carried out at the linear parts of the enzyme reaction. The NADH-TR activity could be shown to be linear for at least 60 min (Fig. 2.4), and thus in some further experiments measurements were carried out at 60 min only. Further characterization of the NADH-TR activity (Table 2.1) confirmed its dependence on the NADH supply and showed that this partial reaction of the respiratory complex I is not sensitive to the various inhibitors of the respiratory complex III and IV (such as Antimycin A and KCN) or of the ATP synthase (Oligomycin). The activity was also not affected by the uncoupler agent FCCP.

The NADH-TR activity of the fibroblasts was further characterized for its membrane association. The Arrhenius kinetics of the activities of the mitochondrial inner membrane respiratory enzyme complexes have been shown to be discontinuous resulting from the change in the activation energies of the enzyme complexes as their membrane lipid environment undergoes a phase change (Marzuki et al. 1975; Raison 1973). The examination of the Arrhenius kinetics of the NADH-TR activity in the normal fibroblasts EIN-2-95 and EIN-3-95, as well as those isolated from the LHON family (EIJbIII-1-94 and EIJbIII-4-94) revealed such discontinuity in the Arrhenius plot (Fig. 2.5). The Arrhenius activation energies were found to be at the range of 12-14 cal/mol and 50-57 cal/mol above and below the discontinuity respectively. The break temperature in the Arrhenius plot, which is the temperature at which the membrane lipid undergoes a phase change (Marzuki et al. 1975; Raison 1973) was found to be at 13-13.5°C, within the same range as that reported for rat liver, beef heart, dog, pig and human kidneys (13-15°C) (Klingenberg et al. 1982; Southard et al. 1983).

Partial Deficiency of the NADH-Tetrazolium Reductase in the Fibroblast Cell Lines from the LHON patients

The microphotometric procedure has been successfully employed to demonstrate a partial deficiency in the activity of NADH-TR in the two fibroblast cell lines which carry the G11778A mtDNA mutation. The measurement of this activity in fibroblasts derived from the LHON patients suggests a lower activity compared to that of the normal fibroblasts (Fig. 2.6a,b). Time course studies confirmed this impression (Fig. 2.4). The NADH-TR activity was found to be 0.30 $A_{510-525}$ unit/60 min and 0.31 $A_{510-525}$ unit/60 min in patients ElJbIII-1-94 and ElJbIII-4-94 compared to 0.43 $A_{510-525}$/60 min in the control fibroblasts. Statistical analysis of the NADH-TR activity on the basis of the measurement from 100 cells/cell line (normal control 0.44±0.11 $A_{510-525}$/60 min and 0.53±0.12 $A_{510-525}$/60 min; patients ElJbIII-1-94 and ElJbIII-4-94 0.33±0.09 $A_{510-525}$/60 min and 0.36±0.11 $A_{510-525}$/60 min respectively) confirms that the difference was statistically significant ($p<0.05$) (Fig. 2.4).

Figure 2.4. NADH-Tetrazolium Reductase Activity in Fibroblast Cells from LHON Patients.

Fibroblast cells of two LHON patients (EIJbIII-1-94 and EIJbIII-4-94) and a normal control (EIN-3-95) were grown on cover slips and stained for the time indicated. The activity was quantitated microphotometrically as in Fig. 2.3 in 100 cells per reaction time and the time course of the reaction is shown in the insert. (O) Normal fibroblast cell line (EIN-2-95), (□) LHON patient EIJbIII-1-94 and (**Δ**) LHON patient EIJbIII-4-94.

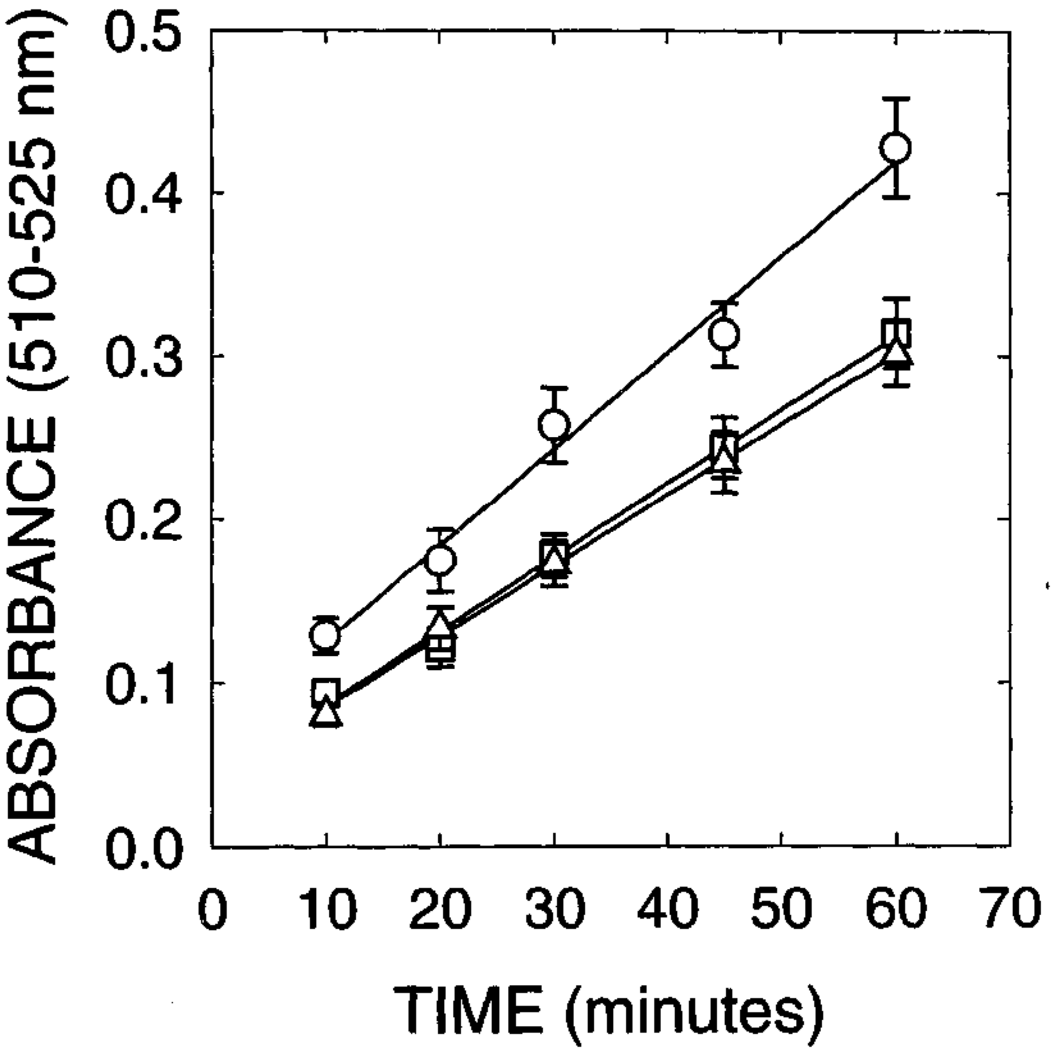

ABSORBANCE (510-525 nm)
0.0
0.1
0.2
0.3
0.4
0.5
0
10
20
30
40
50
60
70
TIME (minutes)

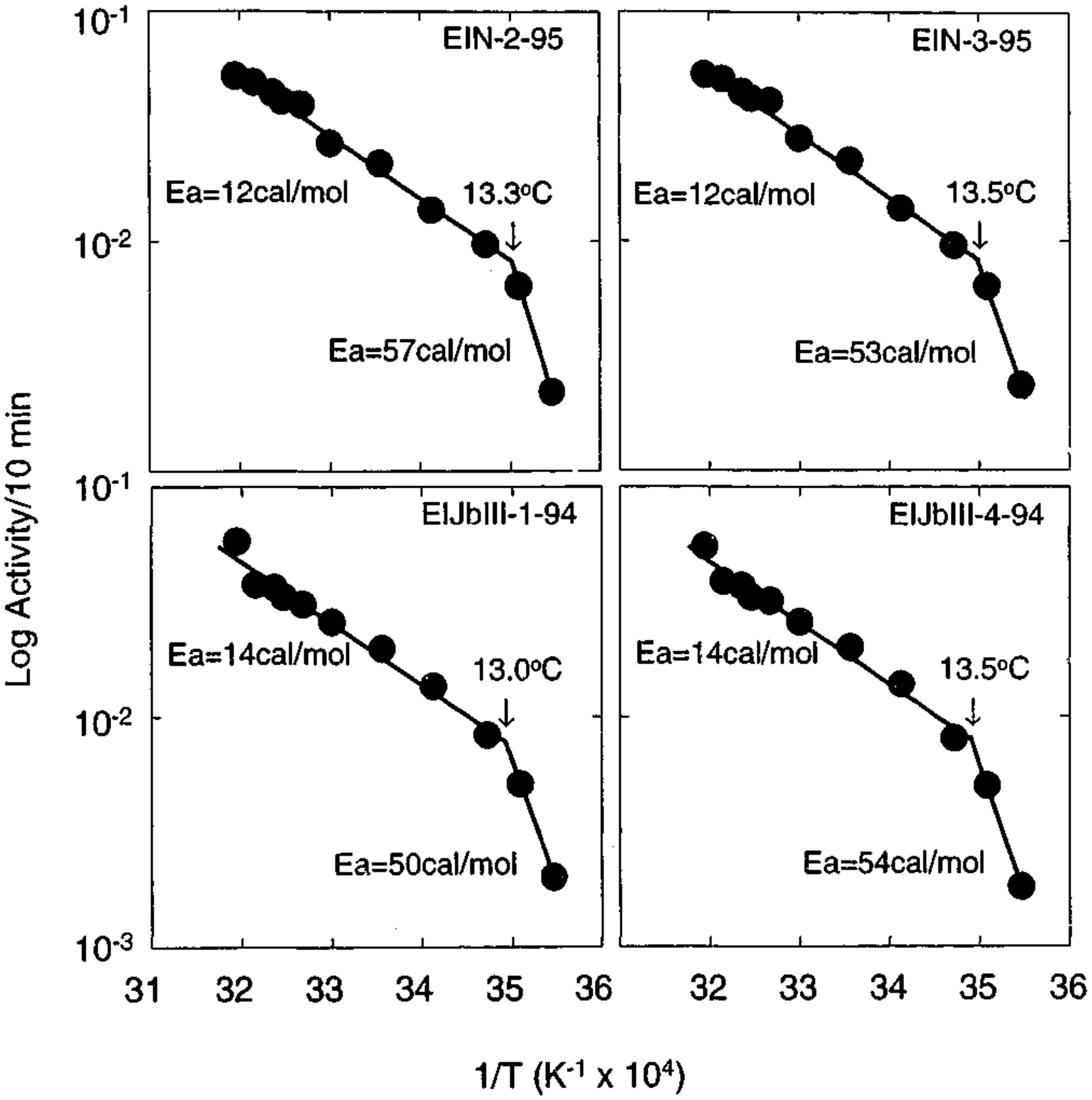
Table 2.1. The Effect of Respiratory Enzyme Complex Inhibitors and Uncoupler to the NADH Tetrazolium Reductase Activity

Assay conditions	**NADH-tetrazolium reductase activity (%)**
Complete reaction	100
Without NADH	0
+ Antimycin A	101.5±11.1
+ KCN	99.6±10.5
+ Oligomycin	100.3±12.8
+ FCCP	99.3±10.3

NADH-tetrazolium reductase activity is expressed as % of the activity in the absence of inhibitor. Results are the Mean ± SD of at least 20 cells with 6 determinations per cell.

Figure 2.5. Arrhenius Kinetics of the NADH-Tetrazolium Reductase Activity.

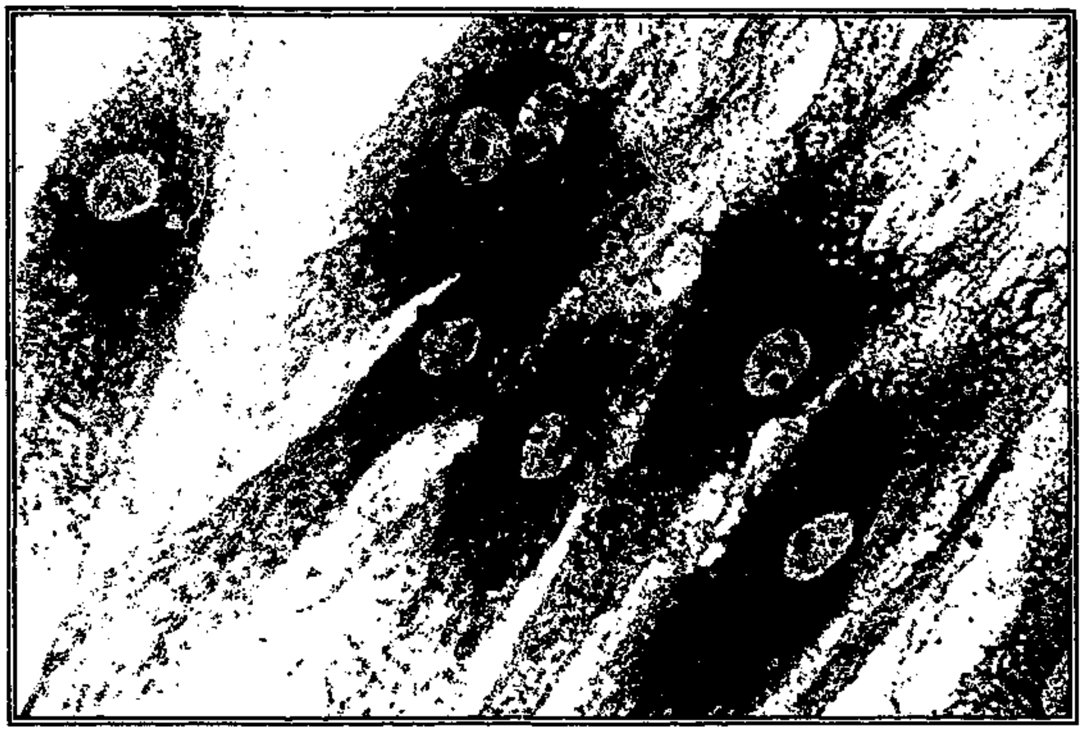
The NADH-tetrazolium reductase activity was determined histochemically as described in Material and Methods, at temperatures ranging from 9 to 40°C (Marzuki et al. 1975; Orian et al. 1984). The NADH-TR activities were shown to be linear for at least 5 h for incubation temperatures of 9, 12 and 15°C, 120 min for incubation temperatures of 20, 25, 27 and 30°C, and 60 min for incubation temperatures of 33, 35, 36, 38 and 40°C. The rate was calculated at the linear part of the curve and expressed as $\Delta A_{510\text{-}525}$ unit/10 min. The intensity of the insoluble blue reduced product was quantitated microphotometrically at 510-525 nm employing a 0.25 mm pinhole as described previously. Arrhenius plots were constructed for the NADH-TR activity. The upper panels are the Arrhenius plots of the normal fibroblast lines (EIN-2-95 and EIN-3-95), and the lower panels are of the LHON fibroblast lines (EIJbIII-1-94 and EIJbIII-4-94). Arrows indicate the break temperatures in the Arrhenius kinetics. The Arrhenius activation energies (Ea) above and below the break temperature are indicated.



EIN-2-95
Ea=12cal/mol
13.3°C
Ea=57cal/mol
EIN-3-95
Ea=12cal/mol
13.5°C
Ea=53cal/mol
EIJbIII-1-94
Ea=14cal/mol
13.0°C
Ea=50cal/mol
EIJbIII-4-94
Ea=14cal/mol
13.5°C
Ea=54cal/mol
Log Activity/10 min
10^{-1}
10^{-2}
10^{-1}
10^{-2}
10^{-3}
31
32
33
34
35
36
32
33
34
35
36
1/T (K^{-1} x 10^4)

Figure 2.6. NADH-Tetrazolium Reductase Activity in Fibroblast Cells from LHON Patients.

Shown are the NADH-tetrazolium reductase staining patterns for the normal fibroblasts EIN-2-95 (top) and for the fibroblasts from patient EIJbIII-1-94 (bottom).



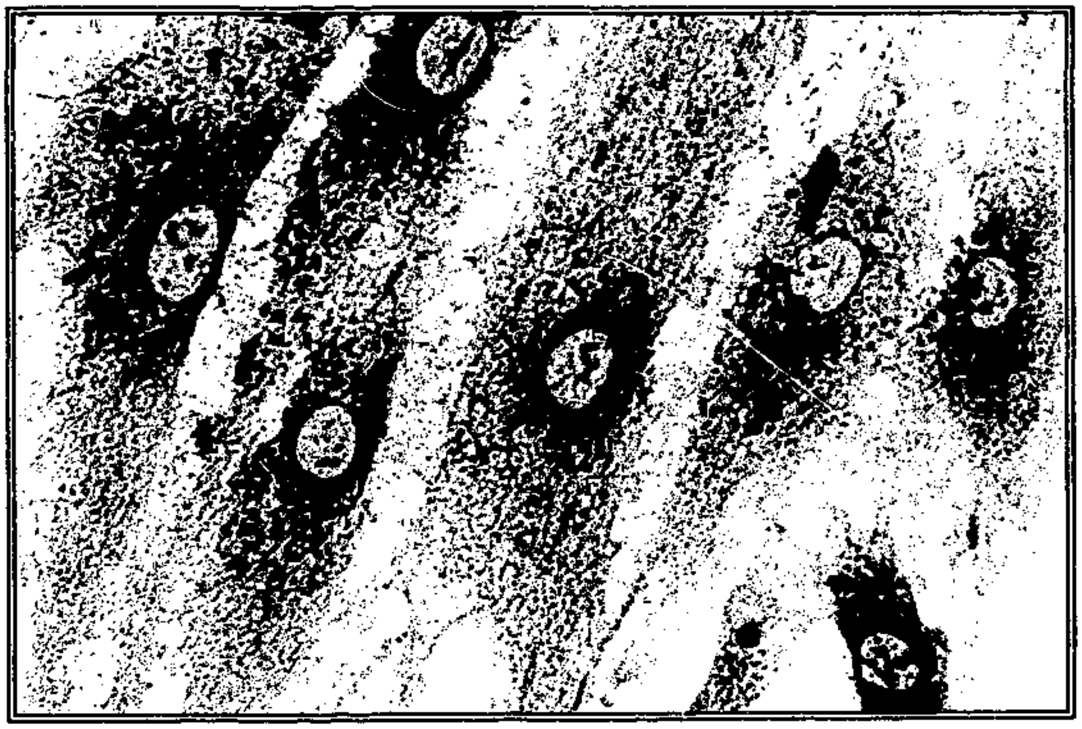
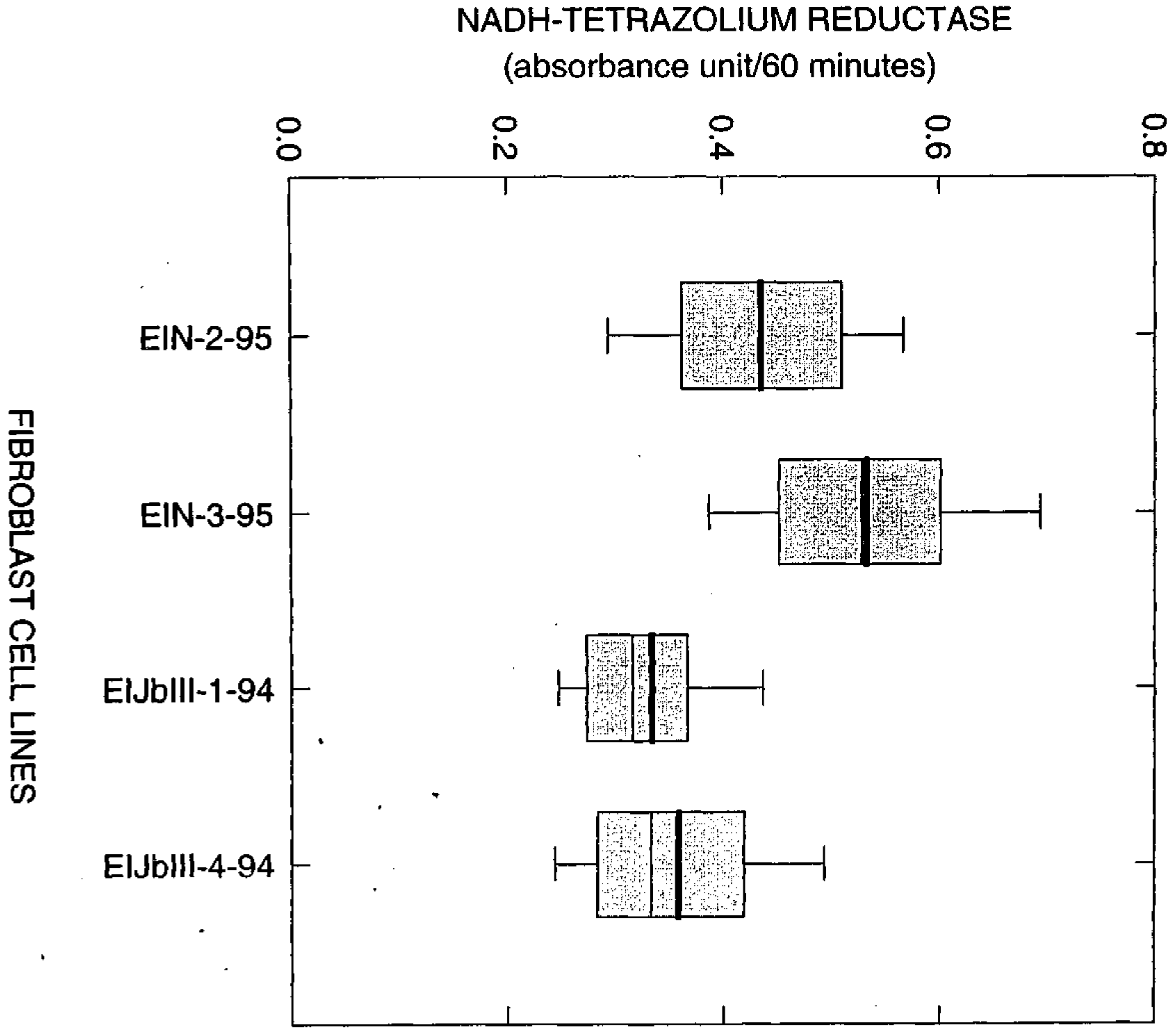


Figure 2.7. Partial Deficiency in Fibroblast Cells Carrying the mtDNA G11778A Mutation.

The distribution of the NADH-tetrazolium reductase activity was studied in fibroblast cell lines from two normal controls (EIN-2-95 and EIN-3-95) and two LHON patients (ElJbIII-1-94 and ElJbIII-4-94). The 25 and 75 percentile coverage is indicated by the boxes, while the error bars indicate 10 and 90 percentile points. The thick and thin horizontal lines within the box are Mean and Median respectively.


NADH-TETRAZOLIUM REDUCTASE
(absorbance unit/60 minutes)
0.0
0.2
0.4
0.6
0.8
FIBROBLAST CELL LINES
EIN-2-95
EIN-3-95
EIJbIII-1-94
EIJbIII-4-94

DISCUSSION

Reports on the deficiency of the respiratory complex I in LHON patients with the mtDNA G11778A mutation have been varied. In two studies, changes in the respiratory complex I activity were reported in muscle (Uemura et al. 1987) and in platelets (Parker et al. 1989) of patients, but the causative mutation in mtDNA was not determined. One study succeeded in demonstrating a decrease in complex I respiratory chain enzyme activity in patients with the mtDNA G11778A mutation, i.e., approximately 25% in the platelet mitochondria (Smith et al. 1994). In contrast, several investigators reported that they have not been able to demonstrate the presence of changes in the activity of the complex I respiratory chain in LHON patients with the mtDNA G11778A mutation, whether in platelets (Majander et al. 1991) or fibroblast cells (Degli Esposti et al. 1994; Sudoyo et al. 1992). For another LHON mutation that affects the ND6 gene, a 60% reduction of complex I activity was reported in EBV-transformed lymphoblastoid cell lines (Jun et al. 1996). Of interest is the observation of an altered *in vivo* skeletal muscle energy metabolism in LHON patients and asymptomatic carriers with G11778A and T14484C mtDNA mutations when assessed by ^{31}P magnetic resonance spectroscopy (Lodi et al. 1997).

One possible explanation for the difference between the present result and that of the earlier studies might be related to the rate limiting step of the respiratory complex I which could be the last step of the reaction, i.e. the electron transfer to the semiquinone intermediate. The NADH-TR reaction is a partial reaction of complex I and although the exact site where electron is captured by NBT is not known, the 340Arg>His amino acid change in the ND4 subunit of the enzyme complex must have affected the electron transfer activity prior to the NBT site (i.e. prior to the rate limiting step of the overall NADH-Coenzyme Q oxidoreductase [EC 1.6.5.3] reaction), and thus might not be detectable as a decrease in the complex I reaction as a whole. Degli Esposti *et al* (1994) suggested that the LHON G11778A mutation increases the sensitivity of complex I to rotenone.

The significance of the decrease in the partial activity of the respiratory complex I in the LHON pathological process is not clear. The observed decrease in activity does not seem to be sufficient to have caused blindness, especially since the partial deficiency in the activity of NADH-TR is not necessarily followed by a decrease in the complex I reaction as a whole. Indeed, the growth rate of the fibroblast cell lines from the two patients did not differ significantly from that of the normal control (data not shown), both in the absence or in the presence of uridine (added because respiratory deficient cell lines cannot grow in its absence; uridine is needed to support *de novo* biosynthesis of pyrimidines via dehydroorotate dihydrogenase which is normally served by the mitochondrial respiratory activity (Desjardins et al. 1986; Gregoire et al. 1984). The possibility must be entertained that the deficiency of the enzyme activity in the optic nerve is far more severe than in cultured fibroblast cells.

Results of the study presented in this chapter demonstrate the utility of the microphotometric analysis in the study of enzyme deficiency at the single cell level. In this study I have used the technique to examine the activity of the mitochondrial NADH-tetrazolium reductase in fibroblast cell lines of patients with Leber's hereditary optic neuropathy. Together with the cytochrome *c* oxidase and succinate dehydrogenase (Collins et al. [illegible], Dubowitz and Brooke 1973), this partial reaction of the respiratory complex I (NADH-Coenzyme Q oxidoreductase) are the most common, and therefore very well characterized, histochemical reactions employed in the assessment of the activity of the mitochondrial respiratory chain in a wide range of neuromuscular disorders. This study has demonstrated that it is possible with the microphotometric approach to obtain sophisticated enzyme kinetic data at the single cell level, including the Arrhenius kinetics of the reaction of interest. This approach could provide information regarding cellular variations in the enzyme activity not otherwise revealed in tissue homogenate. Such information is important, for example, when mosaicity is expected in the expression of a pathological condition, such as that resulting from a mutation in the mtDNA. In this respect, it is interesting to note that while the NADH-TR activity in the two normal fibroblast cell lines show a normal distribution, the activity in both cell lines from the LHON patients significantly demonstrate skewing to the higher activity, i.e. toward the activity in the normal cell lines (Fig. 2.7). Thus, while the means of the activity for EIJbIII-1-94 and EIJbIII-4-94 were 0.324 and 0.359 $_{A510-525}$/60 min, the medians were found to be 0.316 and 0.333, respectively. One possible explanation for this observation is that there is a progressive lost of the respiratory complex I activity in the patient's mitochondria, perhaps due to the relative instability of the assembled enzyme complex; such instability might be more severe in the affected tissues.

CHAPTER 3

CONGENITAL NON-SYNDROMIC DEAFNESS IN A BALINESE FAMILY ASSOCIATED WITH THE A1555G MUTATION IN THE MITOCHONDRIAL SMALL SUBUNIT RIBOSOMAL RNA

INTRODUCTION

The role of mtDNA mutations as the causal molecular defects in a wide range of human disorders is now well established. It has become apparent in recent years, however, that mtDNA associated disorders behave more like polygenic diseases in that the expression of the causal mutation is frequently modulated by their nuclear and mitochondrial DNA backgrounds as well as by environmental factors. The interplay between these factors is most apparent in the sensorineural hearing loss associated with the mtDNA A1555G mutation (Prezant et al. 1993) in a highly conserved region of the small rRNA gene affecting a site in which aminoglycoside resistance mutations have been described in other species (De Stasio and Dahlberg 1990; Montandon et al. 1986; Etzold et al. 1987). The phenotypic expression of the causal mtDNA mutation in this case is precipitated by exposure to aminoglycoside antibiotics, although such exposure is not essential for the phenotypic expression. The involvement of a nuclear recessive factor(s) is indicated by the low penetrance of the causal mtDNA mutation in families with the non-aminoglycosides induced deafness (Prezant et al. 1993; Matthijs et al. 1996), and has been demonstrated experimentally in transmitochondrial cybrids (Guan et al. 2001).

The influence of the overall mtDNA genetic background in the expression of the A1555G mtDNA mutation is less understood. On the one hand, the A1555G mutation is found along distantly related mtDNA backgrounds (Hutchin and Cortopassi 1997; Abe et al. 1998; Estivill et al. 1998), and there is no indication of clustering of the A1555G mutation on any mtDNA haplogroup (Abe et al. 1998; Torroni et al. 1999). On the other hand, there are families with the A1555G mutation in which the chemical expression of the mutation is either exclusively aminoglycoside induced (Prezant et al. 1993; Estivill et al. 1998; Usami et al. 1997; Pandya et al. 1997; Gardner et al. 1997) or congenital/progressive without exposure to antibiotics (Prezant et al. 1993; Matthijs et al. 1996; Estivill et al. 1998; Iwasaki et al.

2000), suggesting the presence of a modifier responsible for precipitating the expression of the mtDNA mutation, which is co-inherited with the A1555G causal mutation. In many cases, the expression of the A1555G in a family are mixed, i.e. antibiotic-induced as well as congenital/progressive deafness without aminoglycoside exposure (Estivill et al. 1998; Usami et al. 1997), superimposing on the mtDNA background.

Most of the sensorineural deafness cases associated with the A1555G mutation that have been reported to date are of the Caucasian (Estivill et al. 1998), or Japanese (Abe et al. 1998; Usami et al. 1997; Iwasaki et al. 2000) mtDNA backgrounds. Our group has reported recently that the mtDNA of Southeast Asians are genetically distant from that of Caucasians and Japanese. Haplotyping studies revealed that at least 60% of the SEA mtDNAs cannot be classified into the established North Asian haplogroups (A, B, F, G, M, M-C and M-D) (Ballinger et al. 1992; Sudoyo et al. 2001), and were shown to be of a novel haplogroup Z and a sublineage of haplogroup M, designated M-a (Sudoyo et al. 2001). Further, a large number of novel sequence variants, not observed previously in Europeans (Caucasians), Africans or Japanese, were found in the ND genes sequence of the mtDNA of the Southeast Asians (62 SNPs in 17 mtDNAs). The genetic and biochemical characteristics of the mtDNA A1555G under the influence of this widely different mtDNA backgrounds, and perhaps also very different nuclear backgrounds, would be of interest.

This chapter reports a seven generation Balinese family with progressive non-syndromic deafness associated with the mtDNA A1555G mutation, which at the first inspection presented itself with a confusing pattern of inheritance. This family has been studied in details to investigate (a) whether the apparently complex pattern of inheritance has arisen because of a multiple entry of the mtDNA A1555G mutation into the pedigree, (b) the clinical expression of the mutation as non aminoglycoside induced sensorineural deafness and its genetic characteristics, and (c) the respiratory enzyme deficiencies as the biochemical expression of the mtDNA mutation. The microphotometric procedure developed in the previous chapter has been employed to assess the NADH-tetrazolium reductase activity. The total mtDNA of members of the family have been sequenced to investigate sequence variants in the coding region and the rRNA genes of the mtDNA of this family.

MATERIALS AND METHODS

Patients

The family members were first examined in Singaraja, on the north coast of the Island of Bali in Indonesia. Other family members were then traced in Denpasar, the capital city 200 km south of Singaraja (Fig. 1a). Thirty seven subjects were analyzed both genetically and clinically. During the examination, blood (10 ml) and hair root samples were taken for DNA study from certain members of the family. These samples were kept at 4°C for about 24 hr before transported to Jakarta. Skin biopsies were also taken from the anterior aspect of the forearm, with informed consent.

Fibroblast Culture

Biopsied materials were treated and cultured as in the previous chapter. The fibroblast cell lines used were derived from two members of the pedigree: one deaf members (EISiV-78-96) and one hearing member (EISiVI-46-96). One fibroblast cell lines derived from normal individual were used as control (EIN-2, Malik et al. 2000).

DNA Isolation and mtDNA A1555G Mutation Detection

Total DNA samples were prepared from blood and fibroblasts as described elsewhere (Lertrit et al. 1992), and subjected to PCR amplification. The analysis of the mtDNA A1555G mutation was performed by amplifying a segment of the mtDNA containing the 12S rRNA gene by employing primer pair L1231 (5'-AACCTCACCACCTCTTGCTCA-3') and H1782 (5'-CTATATCTATTGCGCCAGG TTTCA-3'), which will give a 551 bp fragment. The PCR amplification was performed for 30 cycles, and the condition employed was denaturation at 95°C for 1 min (95°C for 5 min at first cycle), annealing at 56°C for 1 min 30 sec and extension 72°C for 2 min 30 sec (72°C for 5 min for final extension). The amplified DNA was then digested with *Alw*26I restriction endonuclease (Promega, USA), and electrophoresed on a 2% agarose gel (Sigma, USA) to detect the presence of the A1555G mtDNA mutation (Fig. 1c).

Direct sequencing of PCR products

For the sequencing of the hypervariable region I of the D-loop, fragment of the mtDNA was PCR amplified employing primer pair L15904 (L15904: 5'-CTAATACACCAGTCTTGTAAACCGGAG-3') and H181 (5'-TAATATTGAA CGTAGGTGCG-3'). For the sequencing of the whole mtDNA genome, overlapping fragments of the mtDNA were amplified by PCR essentially as described by Marzuki *et al* (Marzuki et al. 1991). The primer pairs employed for this purpose were: L15904 (5'-CTAATACACCAGTCTTGTAAACCGGAG-3') - H1074 (5'-CCCAGTTT GGGTCTTAGCTA-3'), L892 (5'-AGCCACCGCGGTCACACGAT-3') - H2343 (5'-CAGGCTTATGCGGAGGAGAATGTT-3'), L2322 (5'-CATTCTC CTCCGCATAAGCCTGCG-3') - H5482 (5'-GGTAGGAGTAGCGTGGTAA GGGCG-3'), L5223 (5'-GGAGGCCTGCCCCCGCTAACCGGC-3') - H6854 (5'-GCCGGTGGGGATAGCGATGATT-3'), L6789 (5'-GGAATAGACGTAG ACACACGAGC-3') - H8945 (5'-ATGGGGATAAGGGGTGTAGGT-3'), L8316 (5'-TTAACCTTTTAAGTTAAA GATTAAGAGAAC-3') - H11942 (5'-GTAGGAGAGTGATATTTGATCAGG-3'), L11580 (5'-CCATCTGCCTAC GACAAAGAGACC-3') - H13200 (5'-TGCGAACAGAGTGGTGATAGCGCC -3'), and L12376 (5'-ACCCTGACTTCCCTAATTCCCCCC-3') - H16115 (5'-GGTGGCTGGCAGTAATGTA CG-3'). The PCR amplified fragments were then purified by QIAquickTM (QIAGEN Inc, USA) and cycle sequencing reactions were performed as indicated by the manufacturer with ABI PRISM Dye Terminator Cycle Sequencing FS Ready Reaction (Applied Biosystems, Perkin Elmer), using L15901 and H16540 for HVR I, and 60 internal primers for total mitochondrial genome. Excess ddNTP labeling was then removed by ethanol precipitation as described in the protocol. Samples were electrophoresed through 4% acrylamide (BioRad, California, USA) gel using a model 377 automated DNA sequencer (Applied Biosystems, Perkin Elmer, USA). Sequence data were then compared with the previously published sequence (Anderson et al. 1981; Andrews et al. 1999).

Haplotype Analysis

Five restriction endonucleases, which define eight polymorphic sites (10394 *Dde*I, 10397 *Alu*I, 16517 *Hae*III, 12408 *Hinc*II, 12408 *Hpa*I, 13259 *Hinc*II, 13262 *Alu*I, 663 *Hae*III), were employed for haplotype analysis of the mtDNA using the procedure previously described (Ballinger et al. 1992; Torroni and Wallace 1994). Together with the COII/tRNA^{Lys} 9-bp deletion, these polymorphic sites characterize the most common haplogroups associated with the Asian populations. Briefly, DNA fragments were PCR amplified employing primer pairs L9773 (5'-CGACGGCATCTACGGCTCAACAT-3') – H11325 (5'-GAGTTT GATAGTTCTTGGGCAGTG-3'), L15760 (5'-CGGAGGACAACCAGT AAG CTACCC-3') – H181 (5'-TAATATTGAACGTAGGTGCG-3'), L12104 (5'-

CTCAACCCCGACATCATTACCGGG-3') – H13425 (5'-GAGTAGTCCT CCTCTTTTTTCG-3'), and L100 (5'-GGAGCCGGAGCACCCTAT GTCG-3') – H815 (5'-GTTTCCCGTGGGGGTGTGGCTAGG-3'). The PCR amplification was performed for 30 cycles of denaturation at 95°C for 1 min (95°C for 5 min in the first cycle), annealing at 56°C for 1 min 30 sec and extension 72°C for 2 min 30 sec (72°C for 5 min for final extension). The fragments were then digested with *Dde*I, *Alu*I, *Hae*III, *Hinc*II, or *Hpa*I restriction endonucleases. For the detection of the 9-bp deletion, the primer pair L8211 (5'TCGTCCTAGAATTAATTCCC-3'-) and H8310 (5'-AGTTAGCTTTACAGTGGGCT-3') were employed to produce a 99 or 90 bp PCR products. The digested and amplified fragments were then separated by electrophoresis in 1-3% agarose gels (Sigma, USA) and detected fluorographically after staining with ethidium bromide.

Histochemical Analysis of NADH-Tetrazolium Reductase Activity

The mitochondrial complex I activity was determined also histochemically by measuring the NADH-tetrazolium reductase activity (Malik et al. 2000) in two fibroblast cell lines derived from the skin biopsies of the Singaraja family members. The procedure described in Chapter 2 was employed for this purpose.

Isolation of Mitochondria from Fibroblast Cells and Mitochondrial Respiratory Enzymes Activities Measurement.

Mitochondria were isolated from fibroblast cells by a modification of the method described by Towers *et al* (1972). Briefly, the fibroblast cells were washed in the chilled isolation medium containing 220 mM mannitol, 70 mM sucrose, 2 mM HEPES, 1 mM EGTA at pH 7.4, with the addition of 0.5 g/L bovine serum albumin (BSA; Sigma, USA) and protease inhibitors (2mM phenyl methyl sulfonil fluoride (PMSF), 5mM para-amino benzamidin (PAB) HCl, 5 mM ε-Amino caproic acid) prior to isolation. The cells were homogenized in the isolation medium in a motor-driven tightly fitted glass-teflon pestle, for approx. 7 strokes. The cell debris and nuclei were centrifuged from the cell homogenate at 1,000 rpm for 5 min, and 3,000 rpm for another 5 min in the Sorvall SS34 rotor. The mitochondria were collected by centrifugation at 12,000 rpm for 10 min in the Sorvall SS34 rotor. The pellet was then washed twice by resuspension in the isolation medium and collection at 10,000 rpm for 10 min in the Sorvall SS34 rotor. All procedures were carried out at 0-4°C.

The measurements of the mitochondrial respiratory enzyme activities were carried out spectrophotometrically with a dual beam UV-visual spectrophotometer (U-3300, Hitachi, Japan). Rotenone sensitive NADH-cytochrome *c* reductase and succinate-cyt *c* reductase activities were assayed as described by Byrne *et al* (1988). The NADH-cytochrome *c* reductase activity was assayed at 37°C in the isolated fibroblast mitochondria in triplicate using 50 μg of mitochondrial protein in one ml reaction mixture containing 25 mM potassium phosphate and 5 mM magnessium chloride at pH 7.2. The reaction was started by the addition of 150 μM NADH, and the rate of the enzyme activity was determine by following the antimycin A-sensitive *c* reduction of cytochrome by the NADH at 550 nm. The succinate-cytochrome *c* reductase activity was assayed at 37°C in triplicate with 50 μg of mitochondrial protein as starter in one ml reaction mixture as above, by monitoring the antimycin A-sensitive reduction of cytochrome *c* by 20 mM succinate.

Table 3.1. List of Internal Primers Used for Sequencing the Mitochondrial Genome

Primers	Tm	Sequence 5' to 3'	Position	Size (bp)	Hibridize to
L100	74	GGAGCCGGAGCACCCTATGTCG	100-121	22	L-strand
H504	74	ATGGGCGGGGGTTGTATTGATGAG	504-481	24	H-strand
L534	66	CCCCATACCCCGAACCAACC	534-553	20	L-strand
H815	80	GTTTCCCGTGGGGGTGTGGCTAGG	815-792	24	H-strand
L892	66	AGCCACCGCGGTCACACGAT	892-911	20	L-strand
H1074	60	CCCAGTTTGGGTCTTAGCTA	1074-1055	20	H-strand
L1581	74	GGAAAGTGCACTTGGACGAACCAG	1581-1604	24	L-strand
H1782	68	CTATATCTATTGCGCCAGGTTTCA	1782-1759	24	H-strand
H1992	70	GTCGCCTCTACCTATAAATCTTCC	1992-1969	24	H-strand
L2055	74	TGCCCACAGAACCCTCTAAATCCCC	2055-2079	24	L-strand
L2322	76	CATTCTCCTCCGCATAAGCCTGCG	2322-2345	24	L-strand
H2569	74	GCCGTTAAACATGTGTCACTGGGC	2569-2546	24	H-strand
L2672	76	GAGGCGGGCATAACACAGCAAGAC	2695-2718	24	L-strand
L2803	78	GAGCAGAACCCAACCTCCGAGCAG	2826-2849	24	L-strand
L2992	70	TGCAGCCGCTATTAAAGGTTCGTT	3015-3038	24	L-strand
H3185	64	TGATATCATTTACGGGGGAAGG	3185-3164	22	H-strand
L3590	72	CCTAGCCGTTTACTCAATCCTCTG	3636-3659	24	L-strand
L3980	64	ACGCACTCTCCCCTGAACTC	4049-4068	20	L-strand
L4476	80	CCCCTGGCCCAACCCGTCATCTAC	4476-4499	24	L-strand
L4955	72	CATAGCAGGCAGTTGAGGTGGATT	4955-4978	24	L-strand
L5223	86	GGAGGCCTGCCCCCGCTAACCGGC	5223-5246	24	L-strand
H5620	62	TCAGTTGATGCAGAGTGGGG	5620-5601	20	H-strand
L5906	74	GTTCGCCGACCGTTGACTATTCTC	5906-5929	24	L-strand
H5993	80	GCCTAGGACTCCAGCTCATGCGCC	5993-5970	24	H-strand
L6257	72	GGAGGCCGGAGCAGGAACAGG	6257-6277	21	L-strand
L6537	72	GACCGCAACCTCAACACCACCTT	6537-6559	23	L-strand
L6789	70	GGAATAGACGTAGACACACGAGC	6789-6811	23	L-strand
L7006	74	ACGACACGTACTACGTTGTAGCCC	7006-7029	24	L-strand
L7432	110	ACATAAAATCTAGACAAAAAAGGAAGGAATCGAACCCCCC	7432-7471	40	L-strand
L7607	68	GGTCTACAAGACGCTACTTCCC	7607-7628	22	L-strand
L7901	60	TGAACCTACGAGTACACCGA	7901-7920	20	L-strand
L8211	56	TCGTCCTAGAATTAATTCCC	8211-8230	20	L-strand
L8316	72	TTAACCTTTTAAGTTAAAGATTAAGAGAAC	8316-8345	30	L-strand
L8471	64	CCTCCCTCACCAAAGCCCAT	8471-8490	20	L-strand
L8655	64	CACCACCCAACAATGACTAATC	8655-8676	22	L-strand
L8907	54	CTTCTTACCACAAGGCAC	8907-8924	18	L-strand
L9380	74	GCGCGATGTAACACGAGAAAGCAC	9380-9403	24	L-strand
L9773	72	CGACGGCATCTACGGCTCAACAT	9773-9795	23	L-strand
L10126	58	GACTACCACAACTCAACGGCTAC	10126-10148	23	L-strand
L10349	68	CCTAGCCCTAAGTCTGGCCTAT	10349-10370	22	L-strand
L10515	66	CTTCTAGGAATACTAGTATATCGC	10515-10538	24	L-strand
L10997	62	AGCCAACGCCACTTATCCAG	10997-11016	20	L-strand
L11288	68	CTACTACTCACTCTCACTGCCC	11288-11309	22	L-strand
L11580	74	CCATCTGCCTACGACAAACAGACC	11580-11603	24	L-strand
L11891	68	GAACTCTCTGTGCTAGTAACCAC	11891-11913	23	L-strand
L12104	76	CTCAACCCCGACATCATTACCGGG	12104-12127	24	L-strand
L12367	76	ACCCTGACTTCCCTAATTCCCCCC	12367-12390	24	L-strand
L12760	68	ATCGGCTGAGAGGGCGTAGGA	12760-12780	21	L-strand

L13145	70	GCCCACTAATCCAAACTCTAACAC	13145-13168	24	L-strand
L13571	64	CTCTCATCGCTACCTCCCTG	13571-13590	20	L-strand
L13977	78	CCTGCCCCTACTCCTCCTAGACCT	13977-14000	24	L-strand
L14241	66	AAGCCCCCGCACCAATAGGAT	14241-14461	21	L-strand
L14438	72	GGATACTCCTCAATAGCCATCGC	14438-14460	23	L-strand
L14820	68	CCAACATCTCCGCATGATGAAAC	14820-14842	23	L-strand
L15196	60	ATCCGCCATCCCATACATTG	15196-15215	20	L-strand
L15481	66	CTCACCAGACCTCCTAGGCG	15481-15500	20	L-strand
L15904	78	CTAATACACCAGTCTTGTAAACCGGAG	15904-15930	27	L-strand
H16115	66	GGTGGCTGGCAGTAATGTACG	16115-16095	21	H-strand
L16204	66	GCAAGTACAGCAATCAACCCTC	16204-16225	22	L-strand
H16540	80	GTGGGCTATTTAGGCTTTATGACCCTG	16540-16508	27	H-strand

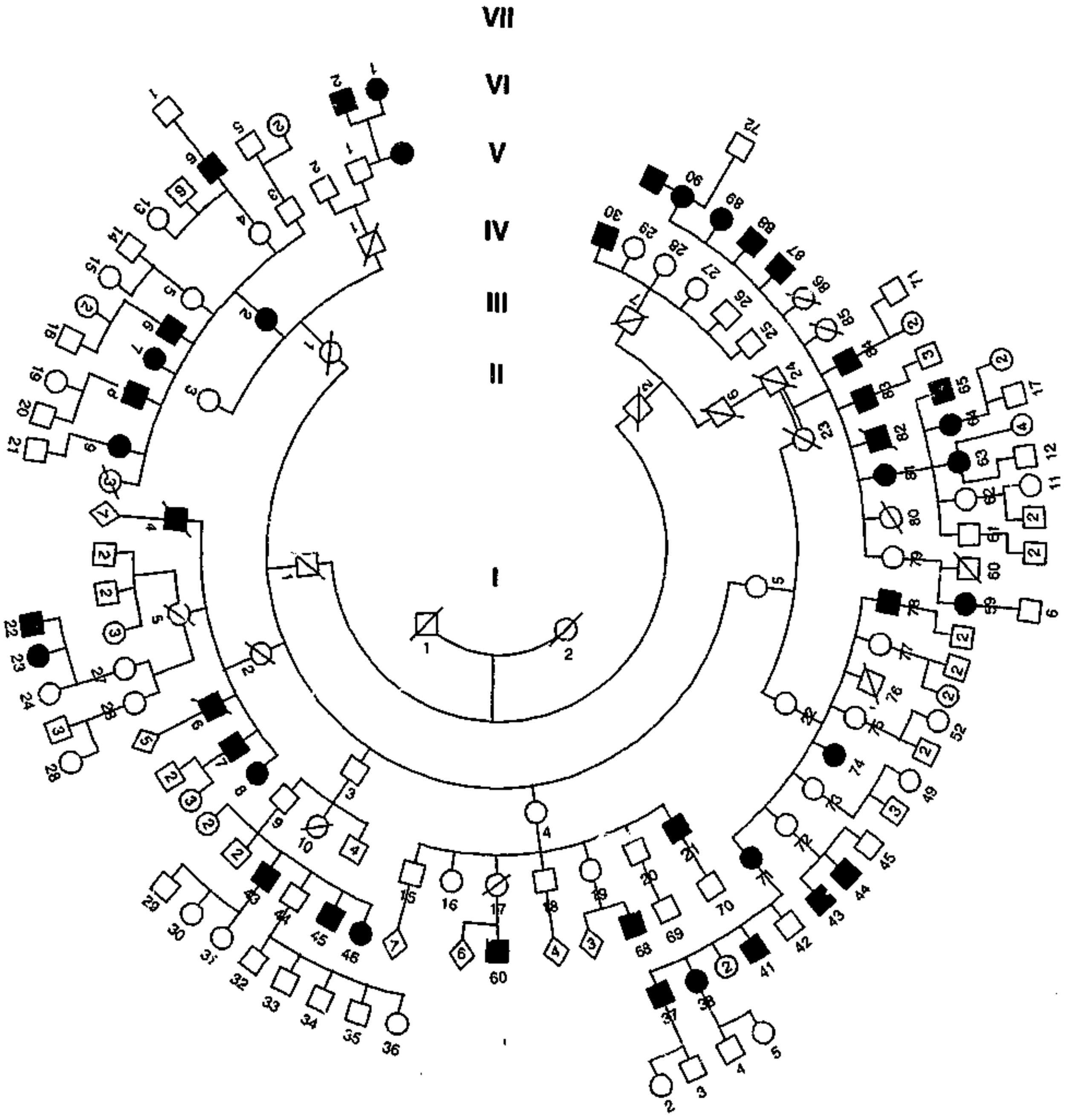
RESULTS

The Balinese family with maternally inherited congenital sensorineural deafness

The large family lived in a village in the Singaraja region of the north coast of Bali, and the pedigree constructed is shown in Fig. 3.1. Deafness can be traced back for seven generations of the 222 family members. Although the pedigree did not show a typical pattern of inheritance, certain branches did suggest a possibility of a maternal mode of inheritance. At the time of the study, there were 133 living members, 37 of whom were available for clinical examination. The hearing examination carried out in a quiet room in the field included tympanometry and pure tone audiometry. All of the 37 patients examined exhibited signs of sensorineural deafness, with clinical manifestation varying from mild to total deafness. The age of onset was found to vary from 2 to 10 years, and extensive interviews established that the Balinese family has no history of exposure to aminoglycoside antibiotics. Local health authorities confirmed that streptomycin has not been used in the region for at least 20 years, while kanamycin and neomycin were only introduced recently and far too expensive for the local population. Neurological examination performed on 20 deaf members excluded other neurological involvement. It was

Figure 3.1. Seven Generation the Balinese Family with Congenital Non-Syndromic Deafness Associated with the A1555G 12S rRNA Mutation.

□■ = male, ○● = female, ■● = affected, □○ = asymptomatic, / = deceased.



VII
VI
V
IV
III
II
I

observed that only 19.4% of the family members exhibited the clinical expression, with a male to female ratio of 26/17.

MtDNA mutation underlying the congenital sensorineural deafness in the Balinese family

The most common mtDNA mutation associated with sensorineural deafness is the A1555G mutation in the 12S rRNA gene. Two deaf members of the family (ElSiVI-43-96 and ElSiV-45-96) were examined for this mutation; direct sequencing of a PCR amplified fragment of the mtDNA revealed the A1555G mutation (Fig. 3.2). The presence of this mutation in a homoplasmic manner was subsequently demonstrated in 35 other members, employing a PCR-based RFLP strategy (Fig. 3.3).

The finding of the mtDNA A1555G mutation is an enigma in term of the pattern of inheritance observed. If it is assumed that the maternally inherited mtDNA A1555G was introduced into the family by the wife of II-1-96, it could explain the deafness transmitted through family members III-1-96, III-2-96, III-4-96 and III-5-96. Deaf family members, however, could be identified also along the paternal IV-1-96, III-3-96 and III-7-96 lines of the pedigree. The mtDNA of family members V-43-96, V-45-96 and V-46-96 who are grandchildren of III-3-96 (male) from IV-9-96 (male) have been examined and found the A1555G mutation also. The hearing wife of IV-9-96, therefore, must have carried the mtDNA A1555G mutation. I did not

Fig. 3.2. MtDNA A1555G mutation in the mtDNA of the Balinese family (1).

A 1451 bp fragment of the mtDNA of family members V-43-96 and V-45-96 containing the 12S rRNA gene was amplified by PCR, employing a set of primers: L892 (5'-AGCCACCGCGGTCACACGAT-3') and H2343 (5'-CAGGCTTATGCGGAGGAGAATGTT-3'). Cycle sequencing reactions were then performed using internal primer L1231 (5'-AACCTCACCACCTCTTGCTCA-3') with ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction (ABI, Perkin Elmer), in a model 377 automated DNA sequencer (ABI/Perkin Elmer). The A1555G mutation is indicated (▼).

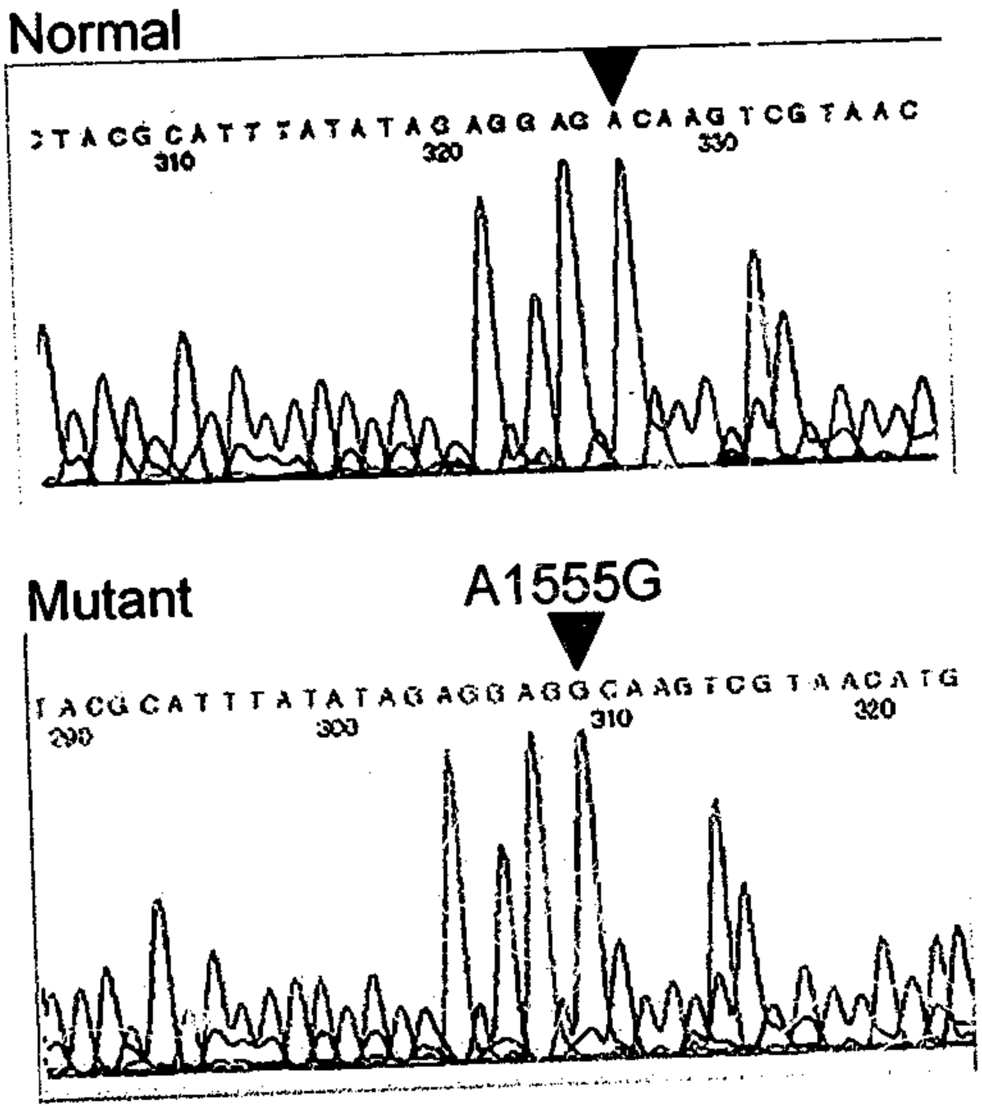
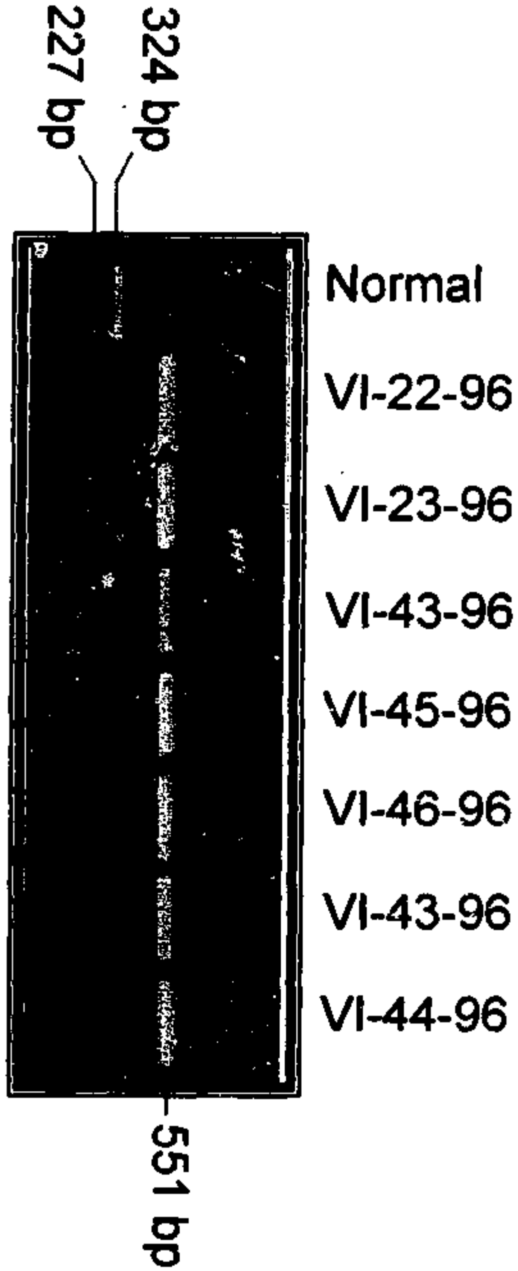

Normal
TACGCATTTATATAGAGGAGACAAGTCGTAAC
310
320
330
Mutant
A1555G
TACGCATTTATATAGAGGAGGCAAGTCGTAACATG
290
300
310
320

Fig. 3.3. MtDNA A1555G mutation in the mtDNA of the Balinese family (2).

The presence of the A1555G mutation was examined in other members of the family (V-46-96, V-71-96, V-73-96, V-74-96, V-77-96, V-78-96, VI-22-96, VI-23-96, VI-37-96, VI-38-96, VI-40-96, VI-41-96, VI-42-96, VI-43-96, VI-44-96, VI-46-96, VI-47-96, VI-48-96 and VI-49-96) by PCR-RFLP using primer pair of L1231 and H1782 (5-'GTACCGCAA GGGAAAGATGAAA-3') and the *Alw*26I restriction endonuclease. PCR amplification was performed for 30 cycles by denaturation at 95°C for 60 sec, annealing at 56°C for 60 sec and extension at 72°C for 120 sec. Shown are RFLP analysis of the PCR-amplified fragment from six members of the family showing the homoplasmic presence of the mtDNA species carrying the mutation.



227 bp
324 bp
Normal
VI-22-96
VI-23-96
VI-43-96
VI-45-96
VI-46-96
VI-43-96
VI-44-96
551 bp

have access to the descendants of IV-1-96 or III-7-96, and thus could not examine the presence of the A1555G mutation in these family members.

To examine whether the mtDNA background associated with the main A1555G mutation in the large family is different from the newly introduced one through the wife of IV-9, the haplotypes of the mtDNA belonging to descendants of III-2 (EISiVI-22-96 and EISiVI-23-96), III-5 (EISiV-78-96 and EISiVI-43-96) and III-3 (EISiV-43-96 and EISiV-44-96) has been investigated by high resolution PCR-RFLP at several diagnostic polymorphic sites in the mtDNA (Torroni et al. 1994; Wallace 1999). All six mtDNA examined were found to be of haplogroup B (absence of 10394 *Dde*I and 10397 *Alu*I sites; gain of 16517 *Hae*III site and the 9-bp deletion) (Torroni et al. 1994) (Table 3.2).

An attempt was made to obtain a more detailed picture of the mtDNA background associated with the two entries of the A1555G mutation by sequencing the highly polymorphic hypervariable region (HVR) I and II of the mtDNA D-loop of the A1555G-carrying mtDNA harboured by the large family (represented by EISiVI-43-96) and that introduced by the wife of IV-9 (represented by EISiV-45-96). Comparison of the sequencing data of the EISiVI-43-96 and EISiV-45-96 revealed that their mtDNA are identical except for one nucleotide difference at nt 186 (G residue in EISiVI-43-96 and C residue in EISiV-45-96). Both types of mtDNA carry 15 and 14 sequence polymorphisms when compared to the Cambridge reference sequence (Table 3.3, Anderson et al. 1981; Andrews et al. 1999).

Table 3.2. MtDNA Haplotypes Observed in Members of the Balinese Family

	10394 *Dde*I	1039 *Alu*I	16517 *Hae*III	9-bp deletion	12406 *Hinc*II	12406 *Hpa*I	13259 *Hinc*II	13262 *Alu*I	663 *Hae*III	Haplotype Group
EISiVI-22-96	-	-	+	+	-	-	-	-	-	B
EISiVI-23-96	-	-	+	+	-	-	-	-	-	B
EISiV-78-96	-	-	+	+	-	-	-	-	-	B
EISiVi-43-96	-	-	+	+	-	-	-	-	-	B
EISiV-43-96	-	-	+	+	-	-	-	-	-	B
EISiV-44-96	-	-	+	+	-	-	-	-	-	B

Table 3.3. SNPs in the mtDNA of the Balinese Family carrying the A1555G mutation

rCRS = revised Cambridge reference sequence [Anderson, 1981 #1087; Andrews, 1999 #1088].

D-loop region

rCRS	73A	150C	186C	196T	263A	309C	309C	441C	522C	523A	16140T	16175A	16182A	16183A	16189T	16217T	16256C	16274G	16335A	16519T
ElSi-VI-43-96	G	T	G	C	G	C+	C+	.	.	.	C	.	C	C	C	C	.	A	G	C
ElSi-V-45-96	G	T	.	C	G	C+	C+	.	.	.	C	.	C	C	C	C	.	A	G	C
ElBal-034-96	G	.	.	C	G	C+	C+	G	del	del	.	.	.	.	.	.	T	.	.	.

tRNAs and rRNA

GENE	F	12S									V	16S										
rCRS	586G	679C	709G	721T	750A	1014A	1385C	1438A	1467C	1555A	1667C	1672C	1693C	1703C	1895C	2239A	2386C	2441C	2534G	2551T	2668A	2670C
ElSi-VI-43-97*	.	.	A	.	G	.	.	G	.	G	.	.	.	.	T	.	.	.	.	.	.	.
ElSi-V-45-97*	.	.	A	.	G	.	.	G	.	G	.	.	.	.	T	.	.	.	.	.	.	.
ElBal-034-96	C	T	.	.	G	T	T	G	T	.	T	T	T	T	.	G	T	T	C	T	C	A

Protein coding

GENE	ND1			ND2		COI		ATP 6	ND4		ND5			Cyt b		
rCRS	3497C	3571C	4155C	4769A	4959G	6978G	7028C	8860A	11334A	11719G	12459A	13220A	13885C	15301G	15326A	15346G
ElSi-VI-43-97*	T	T	.	G	.	C	T	G	.	A	.	G	.	A	G	A
ElSi-V-45-97*	T	T	.	G	.	C	T	G	.	A	.	G	.	A	G	A
ElBal-034-96	.	.	A	G	C	.	.	G	C	A	G	.	A	.	G	.

rCRS = revised Cambridge reference sequence [Anderson, 1981 #1087; Andrews, 1999 #1088]

Sequence analysis of the Balinese family mtDNA that form the background for the A1555G

In a recent report it is described that the SEA mtDNAs are phylogenetically distant from those of the Caucasians (Sudoyo et al. 2001). To investigate the extend of the difference in the mtDNA sequence that forms the background for the A1555G mutation in the Balinese patients, as compared to those of the Caucasian and Japanese, the coding regions of the mtDNA of EISiVI-43-96 and EISiV-45-96 have sequenced, which together with the D-loop sequence in the previous section provide the complete mitochondrial genome sequence of the Balinese patients. In addition, a normal Balinese EIBal-034-96 was also included in this analysis. A total of 15 single nucleotide polymorphic sites (SNPs) were observed in the coding region of the mtDNA of EISiVI-43-96. EISiV-45-96, descendant of III-3, shows an identical set of 15 SNPs (Table 3.3). Of the 15 SNPs in the coding region, seven were found to be novel, not reported previously in Caucasians and Japanese including one in the 16S rRNA gene. Four other SNPs were identified in the rRNA genes (Table 3.3). One of the variant in the variant found in the ND4 gene, 11719A, was also found in the mtDNA of the normal Balinese EIBal-034-96.

Phenotypic Expression of the A1555G Mutation in Fibroblast Cell Lines

The finding of the many mtDNA SNPs set in the Balinese non-aminoglycoside induced deafness patients not previously observed in Caucasian or Japanese, raised the possibility that the significantly different mtDNA background might modulate the expression of the A1555G mutation differently. The defects in the respiratory chain activity as the consequence of the A1555G mutation have thus investigated in fibroblast cell lines derived from symptomatic member ElSi-V-78-96 and asymptomatic member (ElSi-VI-46-96). First, the partial reaction of the respiratory complex I was assessed by employing a well characterized NADH-tetrazolium reductase (NADH-TR) histochemical assay system (Dubowitz and Brooke 1973; Collins et al. 1991) and a microphotometric quantitation procedure (Malik et al. 2000). The result obtained indicates more than 10% reduction of the NADH-TR activities in both ElSi-V-77-96 and ElSi-VI-46 fibroblast lines when compared to a normal cell line (ElN-2-95) (Table 3.4). Fibroblast cells derived from symptomatic member ElSi-V-78-96 grown in the presence of Streptomycin showed a more significant reduction of the NADH-TR activity (21%; Table 3.4).

Further biochemical study was performed to examine the extend of the respiratory complex I activity reduction, since the histochemical assay only measures partial reaction of the NADH-CoQ oxidoreductase. The NADH-cytochrome *c* reductase activity of mitochondria isolated from fibroblasts of the symptomatic member ElSi-V-78-96 showed a significant (35%;

Table 3.4. Respiratory Enzymes Activities Measured in Mitochondria Isolated from Fibroblast Cells with the A1555G mtDNA Mutation

Cell Line	NADH-tetrazolium reductase (% of relative absorbance)		NADH-cyt *c* reductase (nmole cytochrome *c* /min/mg protein)	Succinate-cyt *c* reductase (nmole cytochrome *c* /min/mg protein)
	No streptomycin exposure	With streptomycin exposure		
EIN-2-95	100.0±16.1	97.5±18.5	207.7±24.5	30.5±8.2
EISiV-78-97 (symptomatic)	87.4±17.0	77.4±8.0	134.4±15.5	37.5±0.7
EISiVI-46-97 (asymptomatic)	88.5±13.7	85.5±13.5	181.6±22.3	28.0±2.8

p=0.0006) reduction of the activity when compared to that of a cell line derived from a normal individual (EIN-2-95). The mitochondrial from the asymptomatic member (EISi-VI-46-96) showed only a 13% reduction (Table 3.4). The succinate-cytochrome *c* reductase activity was also examined; no significant difference was observed in the enzyme activity in both A1555G carrying cell lines when compared to the normal cell line (Table 3.4).

DISCUSSION

The study of the large Balinese family has provided us with some interesting observations regarding the genetic characteristics and the biochemical expression of the A1555G mutation under the influence of a very different Southeast Asian mtDNA background. First, it has been able to show that the inheritance pattern of the mtDNA A1555G mutation, which appears to be complex in the first instance, is the result of an unusual multiple entry of the mutation into the pedigree. The pedigree have been cross-checked extensively to ensure that the pattern observed is correct and confirmed it from different branches of the family. Further, it is shown that while maternally related members of the main family (represented by EISiVI-22-96, EISiVI-23-96, EISiV-78-96 and EISiVI-43-96) and descendants of the male member III-3-96 and his son IV-9-96 (represented by EISiV-43-96 and EISiV-44-96) share the same mtDNA haplotype B, D-loop sequence did show one base pair difference indicating that the wife of IV-9-96 is still related to the large family, although perhaps rather remotely. This finding is consistent with the sociocultural setting of the Balinese village, in that most of the family members have been living in a relatively isolated village for generations, and that distance consanguinity through inter-marriage is common.

Secondly, it has also been reported the sequence of three complete Balinese mtDNA, which are the first mitochondrial genome sequences reported for the Southeast Asians. Comparison of variations of the mtDNA sequence of the Balinese family with those of Caucasians and Japanese confirms the genetic distance of the SEA populations (data not shown).

Third, it is shown that despite the phylogenetic distance, the genetic characteristics and biochemical expression of the A1555G mutation is basically similar to those in the Caucasian and Japanese. Thus, while the mtDNA of the Balinese family is found to be homoplasmic for the mutated species in all maternally related members of the family examined, only 19.4% of the family members carrying the mutation exhibited the clinical expression of the mutation, with a male to female ratio of 26/17, which is within the range observed in Caucasian pedigrees (17% to 65% penetrance and 1/3 to 10/5 male/female ratio, Estivill et al. 1998). The age of onset in this family is extremely variable, from congenital deafness in an approx. 5% of the deaf family members to an early onset at the age of 5 years in 35 members examined. Further, examination of the respiratory enzyme activities also revealed a significant decrease in the respiratory complex I activity in particular in the symptomatic members, as observed in an Arab-Israeli pedigree (2001; Guan et al. 1996). Thus, histochemical analysis of the respiratory complex I activity revealed a significant decrease in the activity of NADH-tetrazolium reductase (NADH-TR) after streptomycin exposure in fibroblast cell line derived from a symptomatic member as compared to the normal control. The decrease in the respiratory complex I activity was further confirmed by the observation of a more significant reduction of the NADH-cytochrome *c* reductase activity measured in the fibroblast mitochondria of a symptomatic member. The mitochondrial NADH-cytochrome *c* reductase activity in the fibroblasts of this symptomatic member (ElSi-V-78-96) is 35% less of that of a normal control fibroblast cell line, compare to a 13% reduction in an asymptomatic member. In comparison, the NADH-CoQ oxidoreductase activity was reported to be reduced by 56% and 50% in the symptomatic and asymptomatic members of an Arab-Israeli pedigree with progressive sensorineural deafness associated with the A1555G mutation. Despite the significant reduction of the NADH-cytochrome *c* reductase activity, the succinate-cytochrome *c* reductase activity did not show any significant reduction, indicating that the reduction of the NADH-cytochrome *c* reductase is mainly due to defective NADH-CoQ oxidoreductase.

Finally, attempt to get some insight into the factors that modulate the expression of the A1555G mutation has been performed. It is well documented that while in some families this mutation is associated with aminoglycoside-induced sensorineural deafness, in others it is expressed as either congenital or progressive sensorineural deafness without aminoglycoside exposure. Since the clinical expression of a mtDNA mutation in a particular family is always consistent, i.e. either aminoglycoside induced or congenital/progressive, the factor(s) involved in modifying the expression of the A1555G mutation must be co-inherited with the mutation, perhaps in the form of other nucleotide sequence variants as the mtDNA background. Although a recent report noted that the A1555G mutation occurred in many populations with various ethnic background and is distributed randomly on different haplogroups (Torroni et al. 1999), the possibility of the involvement of a mtDNA secondary mutation(s) which is/are not haplogroup specific could not be discounted.

Thus, the mtDNA 12S rRNA sequences of three families with pure congenital/progressive sensorineural deafness for which the sequence information is available, i.e. the Balinese, an Arab-Israeli family (Prezant et al. 1993), and a Zairean large pedigrees (Matthijs et al. 1996) have been compared. Of the 11 SNPs observed in the two rRNAs of the three families, two are normal variants common in all populations (A750G and A1438G). An SNP, 709G, was found in two of the three non-aminoglycoside induced deafness families (Balinese and Arab-Israeli) (Table 3.5). A further study to examine the involvement of the this variant in the disease process showed that it was also observed in 21%, 24% and 20% of the mtDNA of the hearing Balinese, Japanese and Chinese populations, respectively.

Of the other SNPs observed in the 12S rRNA genes, only three were also found in two families; 769A, 825A and 1018A, all in the Arab-Israeli and the Zairean families. None of these three sites are conserved in mammals (Prezant et al. 1993; Matthijs et al. 1996), and thus are unlikely to be involved. Sequencing data from more pedigrees with exclusive expression of the A1555G mutation as either aminoglycoside induced or non-induced congenital/progressive are needed to elucidate the role of mtDNA modifying factor(s) on the expression of the A1555G.

Table 3.5. Mitochondrial rRNA gene Mutations in Families with Nonsyndromic Sensorineural Deafness

	Non-aminoglycoside induced deafness			Prevalence in normal populations (%)			
GENE	**Balinese (1)**	**Arab-Israeli (1)**	**Zairean (1)**	**Balinese (n=80)**	**Chinese (n=50)**	**Japanese (n=33)**	**Caucasian (n=17)**
12S rRNA							
	G709A	G709A		21	20	24	6
	A750G	A750G	A750G	750G is the major variant found in the population			
		G769A	G769A				
		T825A	T825A				
		A851G					
		G930A					
		G1018A	G1018A				
			T1420C				
	A1438G	A1438G	A1438G	1438G is the major variant found in the population			
	A1555G	A1555G	A1555G				
16S rRNA							
		T1822C					
	C1895T						

CHAPTER 4

NUCLEAR MITOCHONDRIAL INTERPLAY IN THE MODULATION OF THE HOMOPOLYMERIC TRACT LENGTH HETEROPLASMY IN THE CONTROL (D-LOOP) REGION OF THE MITOCHONDRIAL DNA

INTRODUCTION

An extensive catalogue of mitochondrial DNA (mtDNA) mutations that underlie a wide range of human diseases now exists, but the processes involved in the generation of these mutations and their pathobiology are still not well understood. An example is the noticeable site variability of mtDNA substitution rate, in that certain sites in the mtDNA have been found to be "hotspots" for mutations (Hasegawa et al. 1993; Wakeley 1993; Excoffier and Yang 1999; Meyer et al. 1999; Stoneking 2000). This site variability superimposes on the high rate of mtDNA evolution, which is 5-10 times higher than that of the nuclear DNA, due to the lack of a major repair mechanism, absence of protection by nucleoproteins, and exposure to high reactive oxygen species (ROS) produced in the mitochondria as the by product of the OXPHOS (for review see Linnane et al. 1989; Wallace 1999). Many questions remain unanswered in relation to this site variability. Why do certain nucleotides mutate more rapidly than others? Are the mutational events at certain sites influenced by neighbouring nucleotides? Do particular mtDNA sequence polymorphisms influence the expression of pathogenic mtDNA mutations? Are nuclear factors involved in determining the rate of mtDNA mutations?

Emphasizing the importance of the above questions is the finding that many of the pathological mtDNA mutations have occurred several times independently along different lineages of mtDNA. The examples include the Leber's hereditary optic neuropathy (LHON) G11778A and G3460A mutations (1995; Brown et al. 1997; Lamminen et al. 1997; Torroni et al. 1997), and the Mitochondrial Encephalomyopathy with Lactic Acidosis and Stroke-like Episodes (MELAS) A3243G (Jacobs and Holt 2000), the latter being expressed clinically also as diabetes mellitus (Mitochondrial Diabetes Mellitus; MDM) with or without neurosensory deafness (Kadowaki et al. 1994; van den Ouweland et al. 1994; Gerbitz et al. 1995).

Sequence polymorphisms in the mtDNA appear to be involved either in the phenotypic expression of the pathological mtDNA mutations, or in their actual generation, as indicated by the clustering of the LHON G11778A mutation on certain lineages of the mtDNA (Brown et al. 1997; Lamminen et al. 1997; Torroni et al. 1997; Sudoyo et al. 2001). Factor(s) encoded in the nuclear DNA could affect the fidelity of the mtDNA replication machinery (Bendall and Sykes 1995; Marchington et al. 1996), as illustrated albeit rather extremely, by the observation of two heterozygous missense mutations affecting the nuclearly coded heart/skeletal muscle isoform of the adenine nucleotide translocator (ANT1) leading to large-scale mtDNA deletions, which underlie an autosomal dominant progressive external ophthalmoplegia (adPEO) (Kaukonen et al. 2000).

The significantly higher rate of mutations observed in the D-loop control region of the mtDNA makes it an ideal region to examine the interplay between factors involved in error generation during mtDNA replication. The D-loop region contains several homopolymeric tracts, and interestingly, some of the longer tracts exhibit length polymorphisms. Of particular interest is the 16189T variant, which is located within and interrupts a homopolymeric tract of cytosines between nt16184 and nt16193 (Bendall and Sykes 1995; Marchington et al. 1996). A T16189C mutation resulting in a homopolymeric tract of ten cytosines is unstable, and length variation of the poly [C] is observed in a heteroplasmic manner (i.e. length morphs of between 8 to 14 cytosines are found in one individual) (Bendall and Sykes 1995).

The genetic characteristics of the above homopolymeric tract in some detail to identify the factor(s) involved in the generation of the length heteroplasmy have been investigated. Here, it is reported evidence that the relative proportion of the various length of the poly [C] (i.e. the pattern of the length heteroplasmy) is maintained in an individual and that the pattern is regenerated *de novo* following cell divisions. The length variation probably occurs as the result of replication slippage, and the present results shows that the extent of this slippage is determined by both the mtDNA sequence polymorphisms, as manifested by mtDNA haplotypes/ haplogroups, and nuclear factor(s), presumably related to the fidelity of the nuclearly coded components of the mitochondrial DNA replication machinery.

MATERIALS AND METHODS

Families, Fibroblast Cell Lines and Tissues

Four families (EIJb-94, EISb-95, EICb-97 and EIPw-97) harbouring the mtDNA T16189C variant along their maternal lines were included in the present study. One of these families (EISb-95) also carries a heteroplasmic T14484C LHON mutation (Sudoyo et al. 1998) while another (EIPw-97) also carries a C16192T mutation interrupting the homopolymeric cytosine tract. One fibroblast cell line (EISbIII-31-95) was derived from a member of family EISb-95. Fibroblast cell lines (EIPwII-06-97 and EIPwIII-10-97) were derived from two members of family EIPw-97. Two additional fibroblast cell lines (EIN-2-95 and EIN-3-95; see Chapter 2) were also used. Blood and skin biopsies were taken with informed consent as part of a diagnostic work up of the patients. Procedures for the biopsy and the generation of fibroblast cell lines are as previously reported as in Chapter 2.

Ten post mortem tissue samples (thyroid gland, liver, diaphragm, psoas, kidney tubules, adrenal gland, heart, cerebral cortex, basal ganglia and white matter) were obtained from each of five normal individuals who died from non pathological causes at the Cipto Mangunkusumo Hospital morgue, Jakarta, Indonesia. Autopsies were carried out according to the standard technical, legal and ethical procedures of the Cipto Mangunkusumo Hospital. Tissue samples were stored individually in small containers at –80°C until used.

DNA Extraction and Detection of the mtDNA T16189C Variant

Total DNA from blood and fibroblast cells were extracted as described elsewhere (Lertrit et al. 1992). DNA from tissues was extracted by an organic extraction method (Sambrook et al. 1989) with modification as follows: a small piece of tissue was homogenized in a tight glass-teflon, in the presence of cell lysis buffer containing 10 mM Tris-HCL (pH 8.0), 25 mM EDTA and 0.5% SDS. To the lysate, 50 µg/ml RNase T (Qiagen GmbH, Germany) was added followed by incubation at 37°C for 30 min. Protein was then precipitated with ammonium acetate and chloroform (1:1). Following ethanol precipitation, DNA was resuspended in a 10 mM Tris-HCl buffer containing 1 mM EDTA (pH 8.0).

A 637 bp fragment of the mtDNA between nt15904 - nt16540 was PCR amplified using the primer pair L15904 (5'-CTAATACACCAGTCTTGTAAACCGGAG-3') and H16540 (5'-GTGGGCTATTTAGGCTTTATGACCCTG-3') in a programmable thermal cycler (Perkin Elmer 9700 GeneAmp System), essentially as described by Saiki *et al* (1985). The PCR was carried out for 35 cycles of denaturation at 95°C for 20 sec (first cycle at 95°C for 5 min), annealing at 62°C for 20 sec, and elongation at 72°C for 1 min. The amplification product was then digested with *MnlI* restriction endonuclease (New England Biolabs, USA) and the fragments separated by electrophoresis on a 2% agarose gel. The T to C base substitution at nt 16189 was detected as the loss of an *MnlI* restriction site (Marchington et al. 1996; Bendall et al. 1996).

Quantitation of the Poly [C] Length Variant

A procedure was developed to quantitate the poly [C] length variants associated with the T16189C polymorphism. A 172 bp fragment spanning from nt16142 to nt16313 of the mtDNA (the poly [C] in the Cambridge reference sequence (Anderson et al. 1981) is from nt16184 to nt16193) was amplified by PCR using the primer pair L16142 (5'-CTTGACC ACCTGTAGTACAT-3') and H16313 (5'-GTACTATGTACTGTTAAGGG-3'). To avoid artifactual length variations due to 1-bp T-overhangs at the 3' end introduced by *Taq* polymerase that would otherwise complicate the analysis of the length of the poly [C], a restriction endonuclease (*Rsa*I, Promega, USA) is employed to trim the 5' and 3' end of the PCR products resulting in blunt end poly [C] containing fragments of 50-56 bp depending on the length of the homopolymeric tract. The relative proportions of the various length morphs were quantitated using a Bio-Rad GS-700 Imaging Densitometer, after the separation of the trimmed PCR products in a 20% polyacrylamide gel (run at 2300 V for 4 hours) and visualization of the

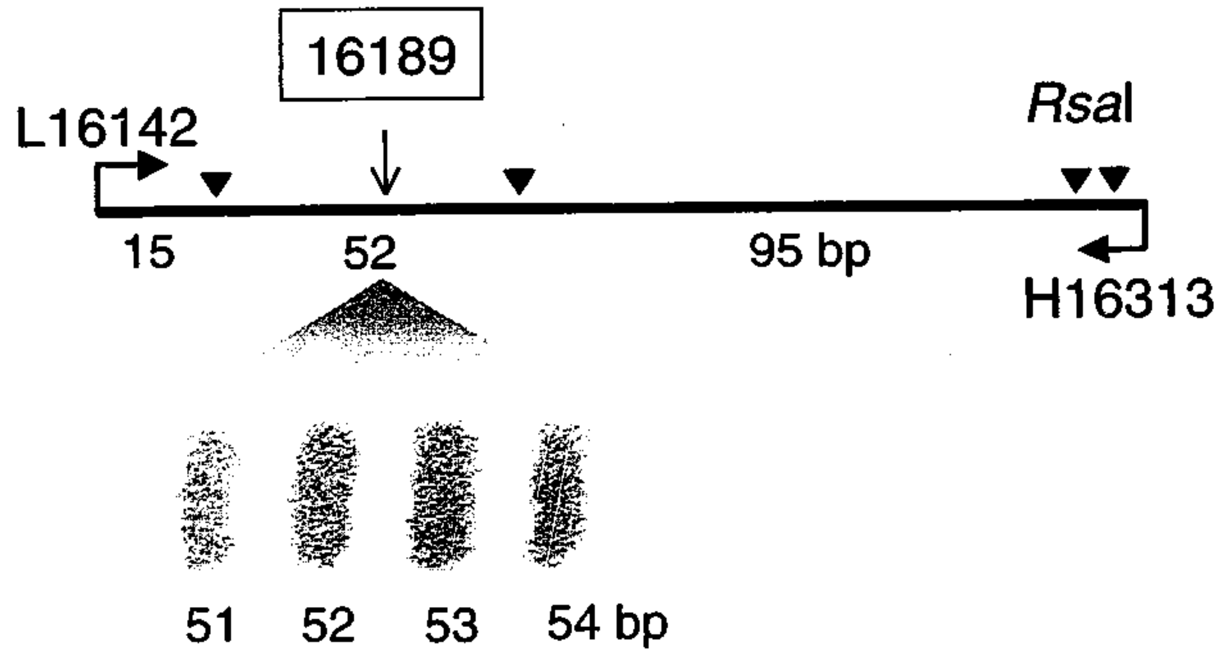
DNA fragments by either silver staining or autoradiography of [^{33}P]-radiolabeled products (Fig. 4.1).

Reduction and Quantitation of mtDNA Copy Number

Fibroblast cells were cultured in the presence of 50 ng/ml ethidium bromide essentially as described previously (Fig. 2, King and Attardi 1989; Vaillant and Nagley 1995). The reduction of mtDNA copy number was assessed during this process by employing a quantitative PCR method, which was carried out with internal standards for both mtDNA and nuclear DNA. The internal standard for the mtDNA is a 1673 bp mtDNA fragment spanning nt2055 – nt3728 which has been inserted into the pGEMT-easy vector (Promega, USA). This fragment carries a PCR-introduced *BglI* restriction site, which is not present in the normal mtDNA, and thus allowing the differentiation of the PCR products of the internal standard from the mtDNA being examined. The internal standard for the nuclear DNA is a pGEMT-easy vector with a 123 bp PCR amplified insert of the α1-globin gene, which has lost the *HinfI* restriction site at codon 116. This mutation is rare and only found in individuals with Haemoglobin O Indonesia (HbO$_{Ina}$), restricted to the populations of South Sulawesi (Daud et al. 2001), and thus should not be present in the samples examined in the current study. Fixed amounts (2 pg) of the internal standards for

Figure 4.1. Determination of the Poly [C] Length Heteroplasmy Associated with the mtDNA 16189C Variant.

The pattern of the poly [C] length heteroplasmy was determined by PCR amplification of a 172 bp fragment spanning nt16142 to nt16313 of the mtDNA and digested by *Rsa*I restriction endonuclease to trim the 5' and 3' end of the PCR products, resulting in the poly [C] containing fragments of 51-54 bp.


16189
L16142
*Rsa*I
15
52
95 bp
H16313
51
52
53
54 bp

mtDNA and nuclear DNA were co-amplified with the DNA samples in serially diluted aliquotes of the fibroblast lysates (Filser et al. 1997; Zhang et al. 1994).

For the quantitation of the mtDNA, a 289 bp fragment was amplified in a Perkin Elmer 9700 thermal cycler employing the primer pair L2055 (5'-TGCCCACAGAACCCTCTAAATCCC-3') and H2343 (5'-CAGGCTTATGC GGAGGAGAATGTT-3'). The amplification was carried out for 28 cycles of denaturation for 20 sec at 95°C (first cycle 5 min at 95°C), annealing for 20 sec at 62°C, elongation for 1 min at 72°C (last cycle 5 min at 72°C), followed by 5 min at 95°C and 5 min at 25°C for final denaturation and slow annealing to reduce heteroduplex formation. The quantitation of the nuclear DNA was performed by using the primer pair HbO and Alf1 (Daud et al. 2001) resulting in a 123 bp fragment. The PCR amplification was carried out for 34 cycles of denaturation at 95°C for 30 sec (the first cycle 95°C for 5 min), annealing and elongation at 70°C for 30 sec (first cycle 70°C for 1 min; last cycle 70°C for 5 min), followed by final denaturation at 95°C for 5 min, and slow annealing at 25°C for 5 min to reduce heteroduplex formation. The PCR products were then digested with either *BglI* restriction endonuclease (Gibco, BRL, USA) for mtDNA quantitation, or *HinfI* restriction endoneuclease (New England Biolabs, USA) for nuclear DNA quantitation. The digested products were separated by electrophoresis on a 2% agarose gel, and the intensity of the fragments were quantitated by densitometric analysis as described earlier. The copy number of the mtDNA was calculated by comparing the intensity of the digested PCR products of the internal standards with the undigested PCR products of the samples being examined for mtDNA quantitation, and vice versa for the nuclear DNA quantitation. The mtDNA copy number quantitation was carried out in triplicates at the exponential stage of the amplification and corrections were made for heteroduplex formation (see Appendix 3).

Single Cell PCR

Fibroblast cells (EISbIII-31-94 carrying the heteroplasmic T14484C LHON mutation and the 16189C variant (Fig. 4.3), and EIN-3-95 carrying only the 16189C variant) were grown in very low cell densities of approx. ten cells per 35 mm tissue culture dish (Nunc, USA). After the first cell division (48-72 hours), the attached cells were washed twice with phosphate buffered saline (PBS, Sambrook et al. 1989) containing a low concentration of 0.0125% (w/v) trypsin. Single fibroblast cells were picked up under an inverted microscope (Nikon, Japan) by suction using borosilicate microcapillary tubes with approx. 3 µm diameter opening, connected to a micromanipulator (Fig. 4.3; Narishige, Japan) (Bidooki et al. 1997). Each isolated cell was delivered directly into a 0.2 ml microfuge tube containing 5 µl sterile double distilled water, lysed at 95°C for 10 min, and subjected to nested PCR amplification.

Figure 4.2. Strategy for mtDNA Reduction and Repopulation.

The mtDNA copy number in a normal fibroblast cell line (EIN-3-95) was extensively reduced to approx. 85 copy/cell, from the original copy number of approx. 3300 copy/cell, by culturing in the presence of 50 ng/ml ethidium bromide for 35 days (approx. 15 cell divisions). The cells were then released from the ethidium bromide exposure to allow the repopulation of the cells with mtDNA for another 35 days (approx. 15 cell divisions).

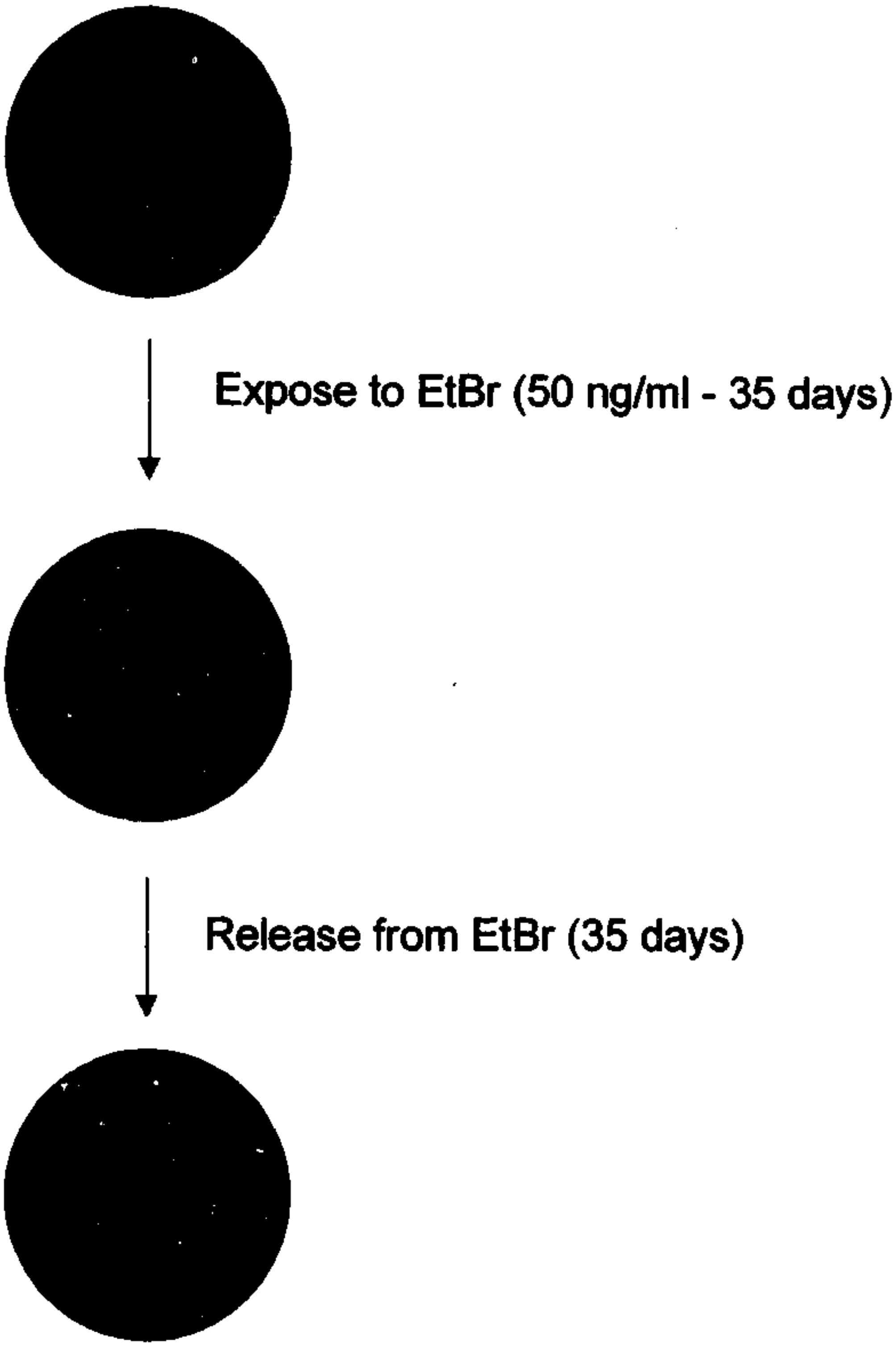
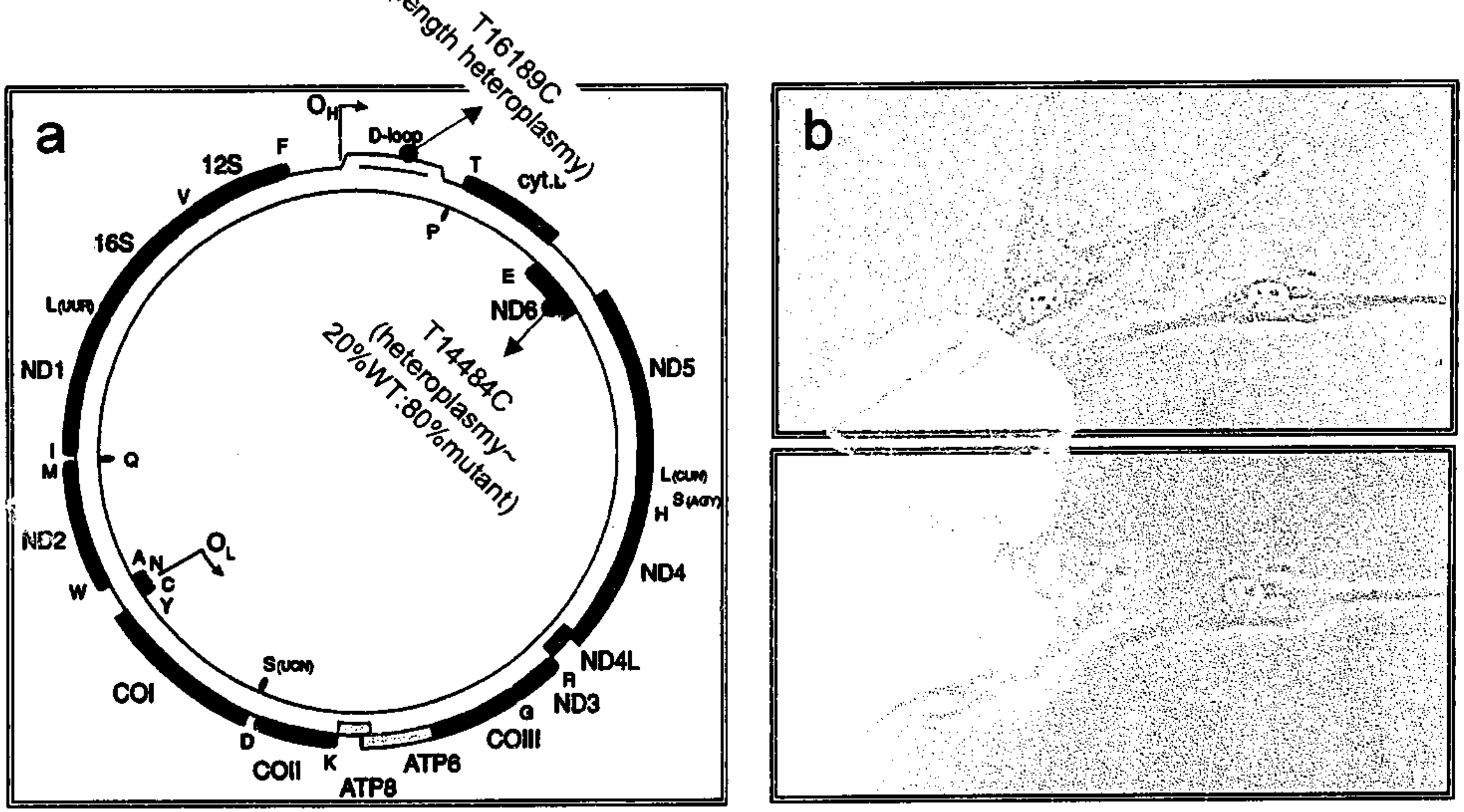

Expose to EtBr (50 ng/ml - 35 days)
Release from EtBr (35 days)

Figure 4.3. Strategy for Random Segregation Determination.

A cell line carrying the 16189C variant and a heteroplasmic T14484C mtDNA mutation as a marker for mtDNA random segregation was employed. Fibroblast cells were grown in very low cell densities of approx. ten cells per 35 mm tissue culture dish. After the first cell division (48-72 hours), single fibroblast cells grown next to each other were picked up by suction using borosilicate microcapillary tubes with approx. 3 µm diameter opening, connected to a micromanipulator. Each isolated cell was subjected to nested PCR amplification.


a
T16189C
(length heteroplasmy)
T14484C
(heteroplasmy~
20%WT:80%mutant)
O$_H$
D-loop
F
12S
V
16S
L(UUR)
ND1
I
M
Q
ND2
W
A
N
C
Y
O$_L$
COI
S(UCN)
D
COII
K
ATP8
ATP6
COIII
G
ND3
R
ND4L
ND4
H
S(AGY)
L(CUN)
ND5
ND6
E
P
T
cyt.b
b

The first PCR amplifies a 637 bp (employing primer pair L15904-H16540) and a 897 bp (employing primer pair L13977: 5'-CCTGCCCCTACTCC TCCTAGACCT-3' and H14873: 5'-GGATCAGGCAGGCGCCAAGGAGTG-3') fragments of the mtDNA. The second PCR to detect the 16189C variant amplifies a 172 bp fragment between nt16142 – nt16313 as described earlier. The T14484C mutation was detected and quantitated essentially as previously described (Sudoyo et al. 1998).

DNA Sequence Analysis

For the sequencing of the hypervariable region 1 (HVR 1) of the mtDNA D-loop, mtDNA fragment was PCR amplified using the primer pair L15904-H16540. PCR products were purified using the QIA quick PCR purification kit (QIAGEN GmbH, Germany), and were sequenced with L15904 and H16540 as the sequencing primers; cycle sequencing reactions were performed as indicated by the manufacturer with BigDye Terminator Cycle Sequencing Ready Reaction Kits and analyzed using a model 377 automatic DNA sequencer (Applied Biosystems, Perkin Elmer).

Haplotype Analysis of the MtDNA

The haplotype of the mtDNA was analyzed by employing five restriction endonucleases, which define eight polymorphic sites (10394 *Dde*I, 10397

*Alu*I, 16517 *Hae*III, 12408 *Hinc*II, 12408 *Hpa*I, 13259 *Hinc*II, 13262 *Alu*I, 663 *Hae*III). Together with the intergenic COII/tRNA^{Lys} 9-bp deletion, these polymorphic sites characterize the most common haplogroups associated with the Asian populations. The procedure employed was essentially as previously described (Ballinger et al. 1992; Torroni et al. 1994). Briefly, DNA fragments were PCR amplified employing primer pairs L9773 (5'-CGACGGCATCTACGGCTCAACAT-3') – H11325 (5'-GAGTTTGATAGTTCTTGGCAGTG-3'), L15760 (5'-CGGAGGACAACCAGTAAGCTACCC-3') – H181 (5'-TAATATTGAACGTAGGTGCG-3'), L12104 (5'-CTCAACCCCGACATCATTACCGGG-3') – H13425 (5'-GAGTAGTCCTCCTCTTTTTCG-3'), and L100 (5'-GGAGCCGGAGCACCC TATGTCG-3') – H815 (5'-GTTTCCCGTGGGGGTGTGGCTAGG-3'). The PCR amplification was performed for 30 cycles of denaturation at 95°C for 1 min (95°C for 5 min in the first cycle), annealing at 56°C for 1 min 30 sec and extension 72°C for 2 min 30 sec (72°C for 5 min for final extension). The fragments were then digested with *Dde*I, *Alu*I, *Hae*III, *Hinc*II, or *Hpa*I restriction endonucleases. For the detection of the 9-bp deletion, the primer pair L8211 (5'TCGTCCTAGAATTAATTCCC-3'-) and H8310 (5'-AGTTAGCTTTACAGTGGGCT-3') were employed to produce a 99 or 90 bp PCR products. The digested and amplified fragments were then separated in agarose gels and detected fluorographically after staining with ethidium bromide.

Cybrids Construction

ElPwII-06[ρ^0] and ElPwIII-10[ρ^0] cells were derived from two fibroblast cell lines (ElPwII-06-97 and ElPwIII-10-97) carrying the 16189C and 16192T variants, by growing the cells in supplemented DMEM (Gibco, USA) as in Chapter 2, in the presence of 50-100 ng/ml ethidium bromide for eight weeks until the cells were completely depleted of mtDNA essentially as previously described (King and Attardi 1989). The presence of mtDNA was detected by PCR amplification of a mtDNA fragment of 636 bp from nt 15904 to nt 16540, and it was performed for 30 cycles of denaturation at 95°C for 1 min (95°C for 5 min in the first cycle), annealing at 56°C for 1 min 30 sec and extension 72°C for 2 min 30 sec (72°C for 5 min for final extension). The amplified fragments were separated in 1.5% agarose gels and visualized under UV light after ethidium bromide staining.

Transmitochondrial cybrids were constructed as described by King and Attardi (1996) and shown in Fig. 4.4. The presumptive transmitochondrial cell lines (cybrid clones) were identified and confirmed by the detection of the presence of the mtDNA and the appropriate β-globin gene polymorphic markers. These markers are polymorphic sites on the β-globin gene located at its intron 2 (Orkin et al. 1982) and 3' end, giving three haplotypes (designated in this study as β-A: *AvalI*-β [+] / *HinfI*-β3' [+]; β-B: *AvalI*-β [-] / *HinfI*-β3' [-]; and β-C: *AvalI*-β [-] / *HinfI*-β3' [+]; Table 4.1).

Figure 4.4. Cybrids Construction Strategy.

The strategy for cybrids construction is as follows: EIPwIII-10[ρ^0] cells were generated from a fibroblast cell line (III-10[III-10]; with two species of mtDNA of eight and nine poly [C]) by the exposure to ethidium bromide as described in Material and Methods. The mtDNA donor fibroblasts (II-06[II-06]; showing only one species of mtDNA with poly [C] length of eight) were enucleated by centrifugation following treatment with cytochalasin B. Cell fusion was induced by the addition of PEG 1450 solution. The presumptive cybrid clones (III-10[II-06]; i.e III-10 nucleus and [II-06] mtDNA) were identified and confirmed by the presence of the mtDNA and the appropriate β-globin gene polymorphic markers (Orkin et al. 1982) (designated β-A: *Ava*II-β [+] /*Hin*fI-β3' [+]; β-B: *Ava*II-β [-] /*Hin*fI-β3' [-]; and β-C: *Ava*II-β [-] /*Hin*fI-β3' [+]).

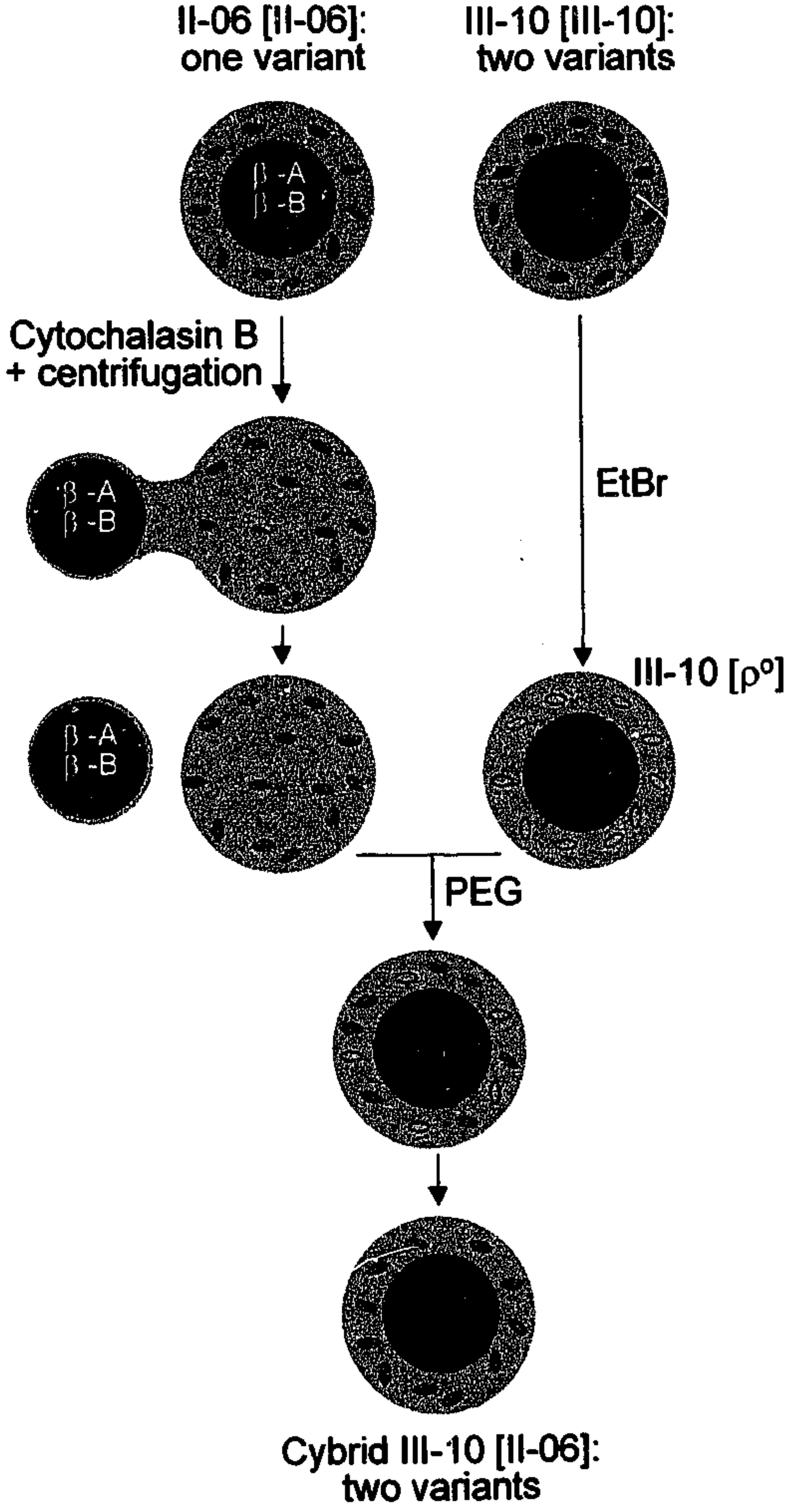

II-06 [II-06]:
one variant
III-10 [III-10]:
two variants
β -A
β -B
Cytochalasin B
+ centrifugation
EtBr
β -A
β -B
β -A
β -B
III-10 [ρ°]
PEG
Cybrid III-10 [II-06]:
two variants

Table 4.1. β-Globin Gene Polymorphic Markers Used to Identified and Confirmed the Cybrid Clones

β-globin haplotype	*Ava*II- β	*Hinf*I- β 3'
A	+	+
B	-	-
C	-	+

The DNA Polymerase γ CAG-Repeat Length Determination

CAG repeats in the 5' end of the catalytic subunit of the DNA polymerase γ gene was examined by PCR amplification using a primer pair and procedures described by Rovio *et al* (1999). The PCR products were separated by electrophoresis in a polyacrylamide gel, visualized by silver staining and analyzed as described earlier. The result was confirmed by DNA sequencing of the region corresponding to nt361- nt487 near the 5' end of the DNA polymerase γ gene.

RESULTS AND DISCUSSION

The Pattern of the mtDNA Length Heteroplasmy is Regenerated De Novo Following Cell Divisions

The main features of the mtDNA length heteroplasmy associated with the T16189C polymorphism are illustrated and confirmed in Fig. 4.5. This polymorphic site is located within and interrupts a homopolymeric tract of cytosines between nt16184 and nt16193. While the sequence of ^{16180}AAAACCCCCTCCCC^{16193} is the most commonly observed mtDNA type, the sequence of ^{16180}AAA$\underline{\text{C}}$CCCCCT^{16189} does occur in some individuals due to A16183C sequence polymorphism in the region. The

16189C morph is associated with the heteroplasmic presence of mtDNA species carrying different length of poly [C] 0f 9 to 15 contained in 51 to 57 bp fragments detected by PCR-restricion enzyme digestion (see Fig. 4.1), containing variation of 9 to 15 cytosines (Fig. 4.5). The relative proportions of the various length of the poly [C] vary between individuals, as represented in four individuals illustrated Fig. 4.6; one with the ^{16180}AAAACCCCCCCCCC^{16193} variant, one with the ^{16180}AAACCCCCCCCCCC^{16193} variant, which associated with additional 16183C variant, and two individuals with the ^{16180}AACCCCCCCCCCCC^{16193} variant, which also carry a further 16182C variant. It is asked whether the variability in the pattern of the length heteroplasmy is simply the outcome of drifts associated with random segregation of the mtDNA population during cell division, or whether this pattern is actively maintained and regenerated *de novo* following each cell division. Three independent experimental approaches were employed. First, the mtDNA copy number in a normal fibroblast cell line (EIN-3-95) was extensively reduced to approx. 85 copy/cell, about 40 times less than the original copy number of approx. 3300 copy/cell, by culturing in the presence of 50 ng/ml ethidium bromide for 35 days (approx. 15 cell divisions). The cells were then released from the ethidium bromide exposure to allow the repopulation of the cells with mtDNA (Fig. 4.2). When the pattern of the length heteroplasmy was examined after 35 days (approx. 15 cell divisions), the pattern was found to be similar as that of the untreated cells (Table 4.2) indicating *de novo* regeneration of the pattern of the length heteroplasmy.

Figure 4.5. Determination of the Poly [C] Length Heteroplasmy Associated with the mtDNA 16189C Variant.

The trimmed PCR products were separated by electrophoresis in a 20% polyacrylamide gel (run at 2300 V for 4 hours) and visualized with silver staining. The relative proportions of the various variants were quantitated using a Bio-Rad GS-700 Imaging Densitometer with Molecular Analyst Software version 2.1.

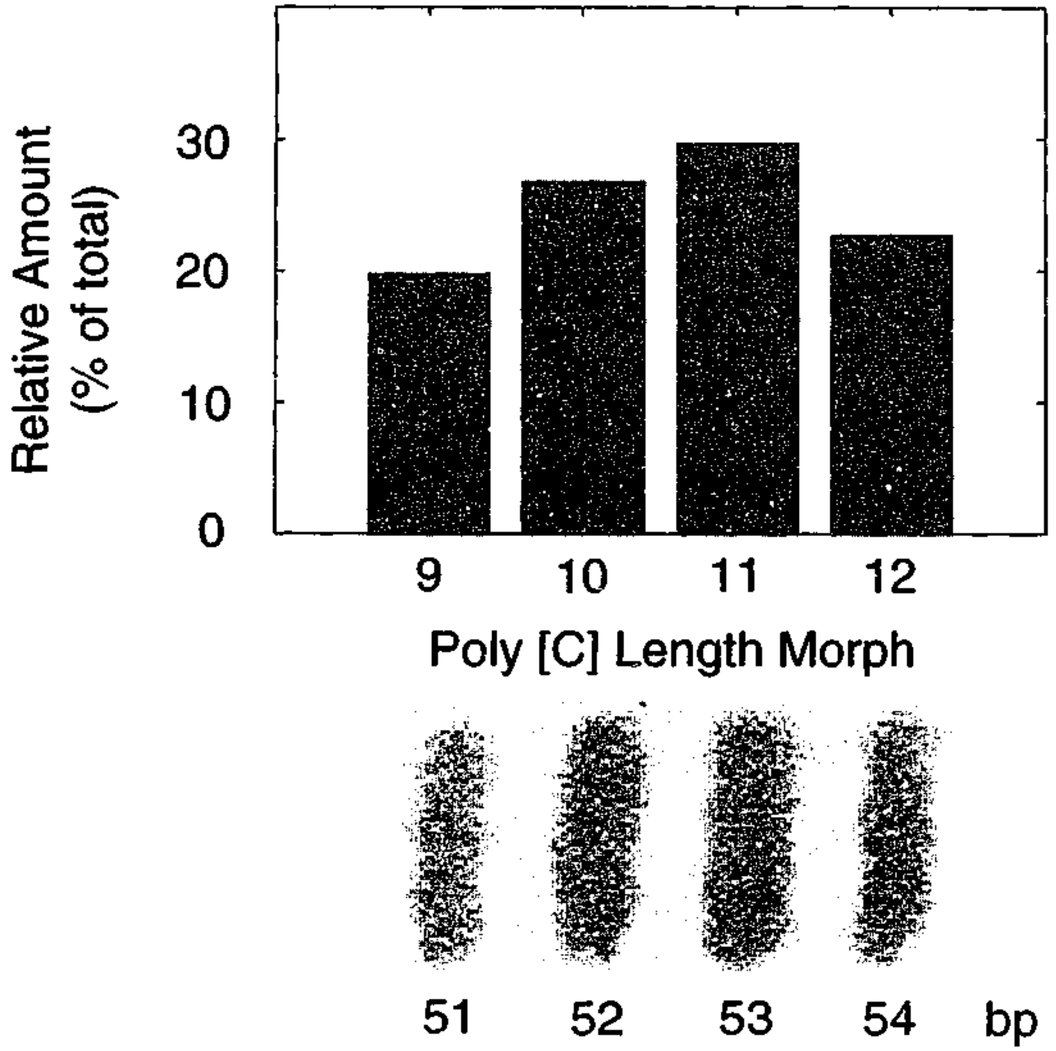

Relative Amount
(% of total)
0
10
20
30
9
10
11
12
Poly [C] Length Morph
51
52
53
54
bp

Figure 4.6. Relative Proportion of the Poly [C] Various Length Associated with the mtDNA 16189C Variant.

Shown are the quantitated proportions of the various length variants (i.e. pattern of the length heteroplasmy) of four individuals showing the ^{16180}AAAACCCCCCCCCC^{16193} variant, the ^{16180}AAACCCCCCCCCCC^{16193} variant, which associated with additional 16183C variant, and two individuals with the ^{16180}AACCCCCCCCCCCC^{16193} variant, both carrying a further 16182C variant.

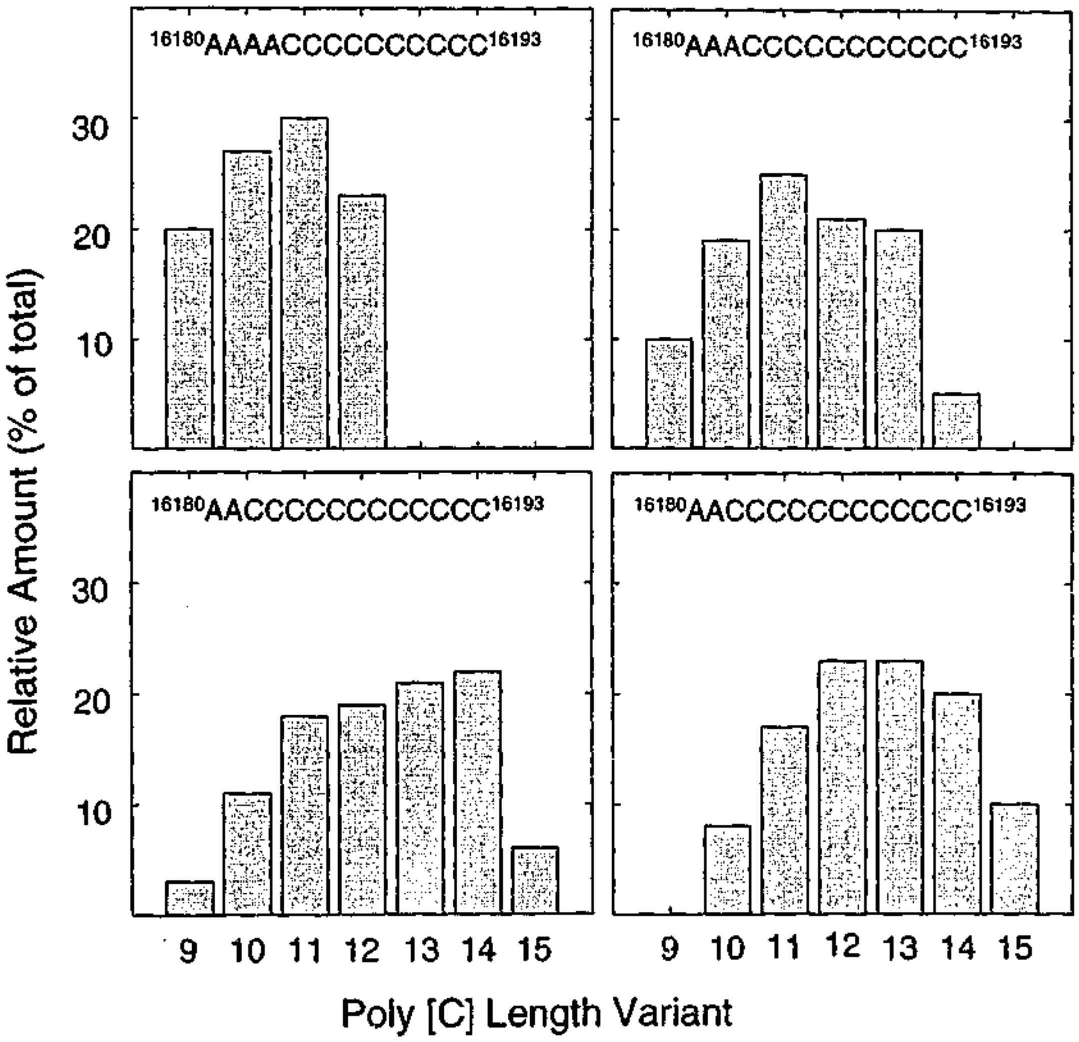

^{16180}AAAACCCCCCCCCC^{16193}
^{16180}AAACCCCCCCCCCC^{16193}
^{16180}AACCCCCCCCCCCC^{16193}
^{16180}AACCCCCCCCCCCC^{16193}
Relative Amount (% of total)
30
20
10
9
10
11
12
13
14
15
Poly [C] Length Variant

Table 4.2. Pattern of Poly [C] Length Heteroplasmy After mtDNA Copy Number Reduction and Repopulation

	Mean Length of Poly [C]	
	EtBr Reduced + Released	Untreated
Experiment 1	11.3 (11.2 and 11.3)	11.3 (11.3 and 11.3)
Experiment 2	11.2 (11.1 and 11.2)	11.3 (11.3 and 11.2)
Experiment 3	11.2 (11.2 and 11.1)	11.4 (11.4 and 11.3)

Results are the average of two independent measurements. Values in brackets are the mean length of poly [C] of each measurement.

To obtain a definitive evidence for the *de novo* regeneration of the pattern of the length heteroplasmy, the pattern was investigated directly in sister cells following cell division by single cell pick up (Fig. 4.7). MtDNA is randomly segregated during cell division, and to allow us to confirm the randomness of the segregation, a cell line, which carries a heteroplasmic T14484C mtDNA mutation, has been employed. Fig. 4.7 shows two examples of such experiment. In the first one, the two sister cells had an almost similar ratio (11:89 and 13:87) of the 14484T to 14484C mtDNA species. The pattern of the length heteroplasmy in the two sister cells was also essentially the same. In the second pair of cells, the random segregation of the mtDNA was observed in a prominent way. Thus, while in one cell the 14484T to 14484C ratio was 46:54, the ratio in the other cell was 100:0. Despite of this significant drift, the pattern of the length heteroplasmy was still essentially the same in the two sister cells. The summary of the results obtained from 12 pair of single cell pick up is presented in Table 4.3. In all cases, the mean length of the poly [C] is essentially the same at an average of 11.4±0.28, despite the fact that the ratio of the 14484T- and the 14484C-carrying mtDNA species varies widely from 100:0 to 0:100. This result provides definitive evidence in support of the earlier suggestion that the pattern of the length heteroplasmy is regenerated *de novo*.

Figure 4.7. Pattern of Length Heteroplasmy in Sister Cells.

Shown are two of twelve pairs examined. In pair I, the two sister cells had an almost similar ratio of the 14484T to 14484C mtDNA species (11:89 and 13:87), and the patterns of the length heteroplasmy are also similar. In pair II, one cell shows a 14484T to 14484C ratio of 46:54, while in the sister cell the ratio is 100:0; despite the random segregation of the mtDNA, the patterns of the length heteroplasmy in the two sister cells remain essentially the same.

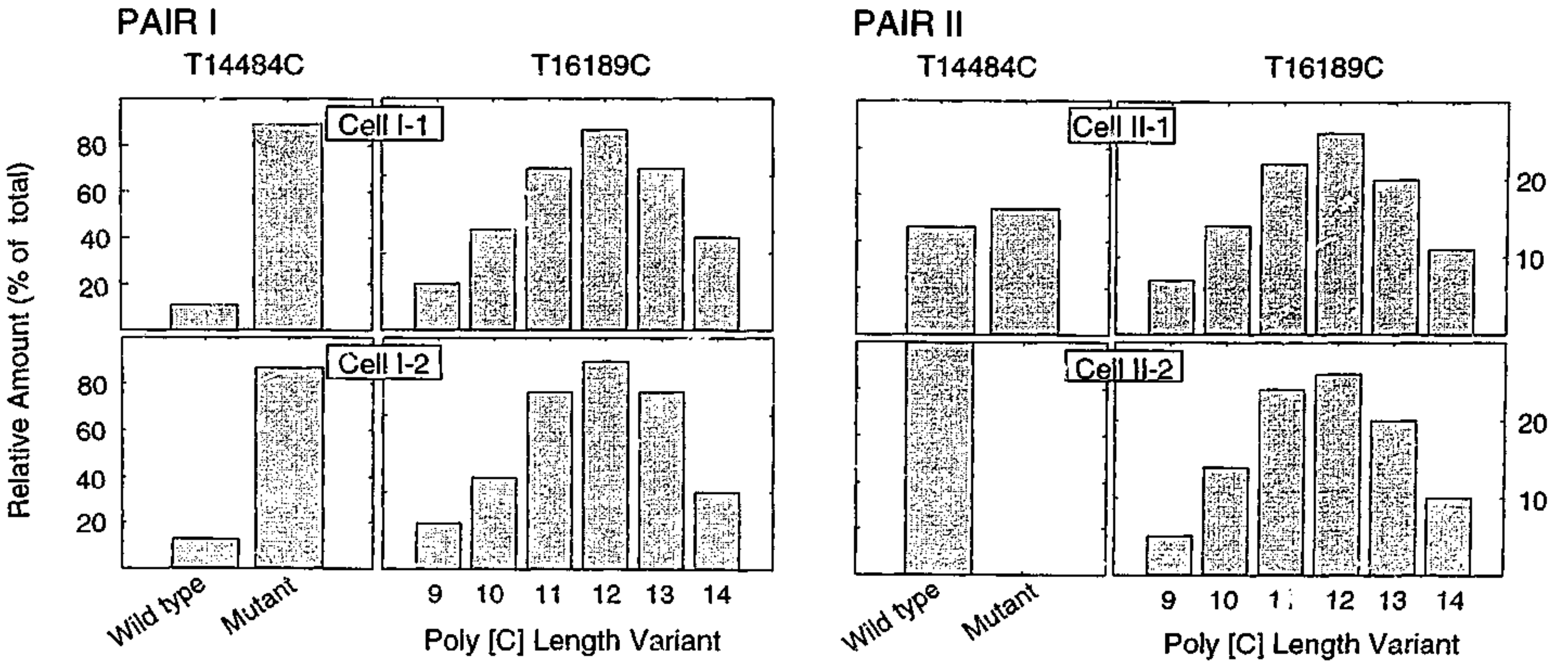

PAIR I
T14484C
T16189C
Cell I-1
Cell I-2
Relative Amount (% of total)
80
60
40
20
80
60
40
20
Wild type
Mutant
9
10
11
12
13
14
Poly [C] Length Variant
PAIR II
T14484C
T16189C
Cell II-1
Cell II-2
20
10
20
10
Wild type
Mutant
9
10
11
12
13
14
Poly [C] Length Variant

Table 4.3. Pattern of Poly [C] Length Heteroplasmy in Sister Cells

	Mean Length of Poly [C]	14484T/14484C (%)
I-1	11.7±0.96	11/89
I-2	11.9±1.09	13/87
II-1	11.7±0.92	46/54
II-2	11.6±1.01	100/0
III-1	11.8±1.20	98/2
III-2	11.4±0.63	16/84
IV-1	11.4±1.14	93/7
IV-2	11.3±1.07	18/82
V-1	11.2±1.20	28/72
V-2	11.6±0.93	21/79
VI-1	11.3±1.16	11/89
VI-2	11.4±0.96	19/81
VII-1	11.4±0.64	21/79
VII-2	11.4±0.72	13/87
VIII-1	10.9±0.83	10/90
VIII-2	11.5±1.03	16/84
IX-1	11.3±0.96	15/85
IX-2	10.8±1.03	0/100
X-1	11.2±0.80	8/92
X-2	11.1±1.00	6/94
XI-1	11.3±1.37	85/15
XI-2	11.1±0.89	5/95
XII-1	11.2±0.75	32/68
XII-2	11.3±1.02	13/87

Result are expressed as the mean length of poly [C]±SD of at least three independent measurements.

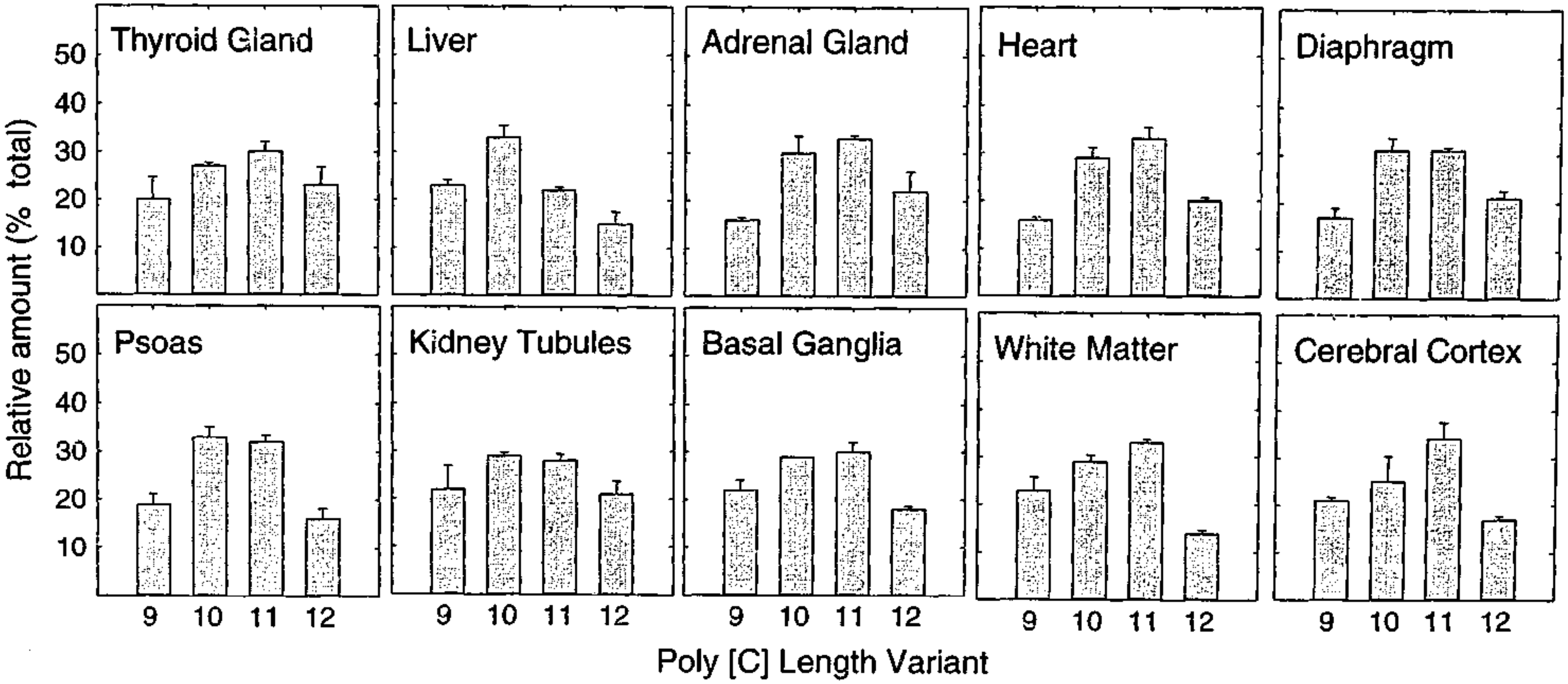
Finally, it was argued that if the pattern of the length heteroplasmy is indeed regenerated *de novo* independent of the random segregation of the mtDNA, the pattern of the length heteroplasmy would then be the same in all tissues of an individual. A limited set of results has been reported that indeed consistent with the above argument (1996; Marchington et al. 1997). To obtain stronger evidence in support of this suggestion, an extensive examination involving ten different post mortem tissue samples (thyroid gland, liver, diaphragm, psoas, kidney tubules, adrenal gland, heart, cerebral cortex, basal ganglia, and white matter) for each of the five individuals examined has been carried out. The pattern of the length heteroplasmy varies between these five individuals, but similar although not completely identical patterns of length heteroplasmy were observed in all tissues of each individual (Fig. 4.8).

Mitochondrial Factor(s) Involvement in the Generation of the Length Heteroplasmy

It was interested to find out whether the factors that determine the pattern of the length heteroplasmy is located within the mtDNA and whether it is also determined by nuclearly coded factor(s). Earlier studies have indicated that the pattern of the length heteroplasmy associated with the 16189C variant is maintained along the maternal lines suggesting that the pattern is determined by factor(s) within the maternally inherited mtDNA

Figure 4.8. The Pattern of the Length Heteroplasmy in Various Tissues.

Ten different post mortem tissue samples (thyroid gland, liver, diaphragm, psoas, kidney tubules, adrenal gland, heart, cerebral cortex, basal ganglia, and white matter) were obtained from each of five individuals at autopsy. The relative proportion of the various length variants associated with 16189C was determined as in the Legend to Fig. 4.1. A representative set of data from one individual is shown. In all cases, a similar pattern of length heteroplasmy is observed in all tissues.


Thyroid Gland
Liver
Adrenal Gland
Heart
Diaphragm
Psoas
Kidney Tubules
Basal Ganglia
White Matter
Cerebral Cortex
Relative amount (% total)
10
20
30
40
50
9
10
11
12
Poly [C] Length Variant

(Marchington et al. 1996; Bendall et al. 1996). The pattern of the length heteroplasmy in three independent families was examined and confirmed that while the pattern is different in the three families, maternally related members of each family show similar patterns of length heteroplasmy (Fig. 4.9).

Sequence polymorphisms in the mtDNA, as indicated by the clustering of some pathological mtDNA mutations on certain mtDNA lineages (haplotype groups; haplogroups), have been suggested to be involved in the expression and generation of pathological mtDNA mutations (Brown et al. 1995). Such polymorphisms could also be the responsible mtDNA factor(s) that maintain the pattern of the length heteroplasmy. This possibility has been investigated by first analyzing the association of specific patterns of the length heteroplasmy with the general mtDNA background as defined by its haplotype group. Of the 35 individuals with the 16189C variant included in this study, 15 (43%) were found to exhibit the ^{16180}AAACCCCCCCCCCC^{16193} variant associated with an additional A16183C polymorphism (modal length of 11Cs; designated the [11C]-group; Table 4.3); the other 57% (20/35) showed the ^{16180}AACCCCCCCCCCCC^{16193} (modal length of 12Cs; designated the [12C]-group) due to a further A16182C polymorphism.

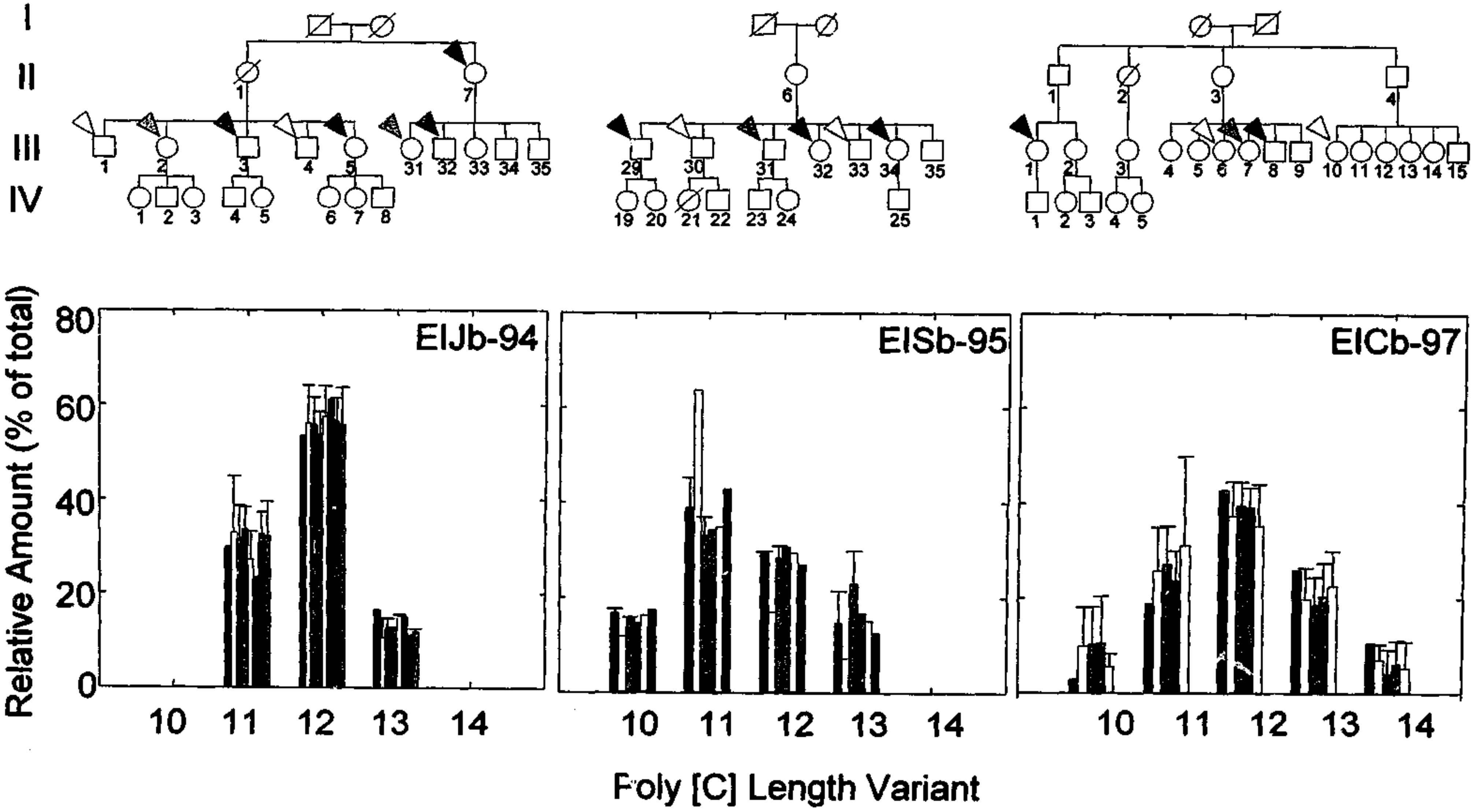
The mean length of the poly [C] and the distribution of the lengths as indicated by the standard deviation were used to describe the pattern of the length heteroplasmy in each individual and to classify the patterns

(Table 4.3). As expected from the earlier results, the mean length of the poly [C] varies widely in both the [11C]- and [12C]-groups. In the [11C]-group, 60% (9/15) of the mtDNA was found to have the short poly [C] mean length of between 11.2-12.0 ([11C]-short), with the rest showing the high mean length of between 12.2-12.8 ([11C]-long). In the [12C]-group, 45% (9/20) of the mtDNA have the short mean length of between 12-12.5 ([12C]-short), while the rest showed the long mean length of above 12.5 up to 13.6 ([12C]-long; Table 4.3).

The haplogroups of the mtDNA are also shown in Table 4.3, as determined by eight polymorphic sites (10394 *Dde*I, 10397 *Alu*I, 16517 *Hae*III, 12408 *Hinc*II, 12408 *Hpa*II, 13259 *Hinc*II, 13262 *Alu*I, 663 *Hae*III), and the intergenic COII/tRNA^{Lys} 9-bp deletion, which together define six of the most common Asian mtDNA.haplogroups (A, B, C, D, F and M, Wallace et al. 1999). Of the total 35 individuals examined, 15 was found to be of haplogroup B (absence of 10394 *Dde*I and 10397 *Alu*I sites; gain of 16517 *Hae*III site and the 9-bp deletion), three of haplogroup B* (characterized by the absence of 10394 DdeI and 10397 AluI sites and gain of 16517 HaeIII site, but lack of the 9-bp deletion, Sudoyo et al. 2001, submitted for publication), ten of a novel haplogroup Z (defined in a parallel study in our laboratory by the gain of the DdeI site at nt 10394 but absence of the related 10397 AluI site, Sudoyo et al. 2001, submitted for publication), four of haplogroup M-a (gain of 10394 DdeI, 10397 AluI and 16517 HaeIII sites, Sudoyo et al. 2001, submitted for publication), and one of haplogroup F (absence of 10394 *Dde*I and 10397 *Alu*I sites;

Figure 4.9. Length Heteroplasmy in Three Matrilineal Pedigrees.

Maternally related members (∇) of three pedigrees (upper panel): ElJb-94 (left), ElSb-95 (center) and ElCb-97 (right) were examined for the pattern of the length heteroplasmy as described in Fig. 4.1. The relative proportions of the length variants associated with 16189C in the various family members are shown under each pedigree.


I
II
III
IV
1
7
1 2 3 4 5 31 32 33 34 35
1 2 3 4 5 6 7 8
6
29 30 31 32 33 34 35
19 20 21 22 23 24 25
1 2 3 4
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
1 2 3 4 5
Relative Amount (% of total)
0
20
40
60
80
EIJb-94
EISb-95
EICb-97
10 11 12 13 14
10 11 12 13 14
10 11 12 13 14
Foly [C] Length Variant

gain of 16517 *Hae*III site; loss of 12408 *Hinc*II/*Hpa*I sites). In addition, two individuals could not be classified into any haplogroup as they did not show any polymorphism at the diagnostic sites.

The longer mean length of the poly [C] in the [12C]-group appears to be associated with haplogroup B. Thus, while almost 73% of the mtDNA with the mean length of more than 12.5 ([12C]-long) in this group was found to be of haplogroup B, only 33% of those with the mean length of between 12-12.5 ([12C]-short) belong to this haplogroup ($p<0.0001$; Table 4.4). Similar haplogroup association was also observed in the [11C]-group. Nearly 67% of the mtDNA with the mean length of between 12.2-12.8 ([11C]-long) was found to be of this haplogroup as compared to 22% of the [11C]-short (mean length of between 11.2-12.0; $p<0.0001$; Table 4.4).

An attempt was made to identify the nucleotide polymorphisms, which could potentially be responsible for the above event by sequencing the region in the mtDNA surrounding nt 16189 (between nt 16000 and nt 16520) in the HVRI of the mtDNA D-loop (Table 4.5). One variant, 16217C, was found to be specifically associated with haplogroup B (72.2%), while another, 16266A, with haplogroup Z (100%). The factors involved in the determination of the mutational hotspots have been extensively studied and reviewed by Rogozin *et al* (2001). Most evidence suggests that mutational hotspots emerge due to neighbouring nucleotides

Table 4.4. Association of Patterns of the Length Heteroplasmy with Haplotype Groups of the mtDNA

	Poly [C] Length		Haplotype
	Mean	SD	Group
AAACCCCCCCCCCC ([11C]-group)			
11-12 C ([11C]-short			
Dmc15	11.2	0.84	Others
Dmc26	11.4	0.89	Z
C-Sd01	11.5	1.40	F
C-15	11.6	0.93	Z
C-61	11.7	1.01	B
CCh064	11.7	0.86	B
CCh048	11.8	0.94	M
CCh042	11.9	1.09	B
C-53	12.0	1.11	M
>12 C ([11C]-long)			
Dmc43	12.2	1.09	B
C-33	12.3	1.44	Z
CCh030	12.5	0.65	Z
CCh049	12.6	1.26	Z
CCh012	12.8	1.42	Others
C-Sd28	12.8	0.97	Z

	Poly [C] Length		Haplotype
	Mean	SD	Group
AACCCCCCCCCCCC ([12C]-group)			
12-12.5 C ([12C]-short)			
CCh031	12.0	1.0	B*
Dmc33	12.3	1.03	Z
CCh022	12.3	1.07	B
C-Sd14	12.3	1.04	M
Dmc56	12.3	0.79	Z
Dmc66	12.5	0.91	B
C-13	12.5	1.25	B
C-Sd11	12.5	1.21	B*
C-Sd25	12.5	1.10	M
>12.5 C ([12C]-long)			
C-Sd18	12.6	0.94	B
Dmc50	12.6	1.04	B
Dmc63	12.6	0.87	B
CCh020	12.7	1.07	B
CCh055	12.7	1.13	B
CCh023	12.9	1.26	B
C-9	13.0	0.63	B
C-25	13.0	1.35	Z
C-Sd29	13.3	0.99	B
C-17	13.5	1.14	Z
CCh051	13.6	1.13	B*

Table 4.5. MtDNA Background Associated with the Pattern of Poly [C] Length Heteroplasmy

Haplogroup	[12C]-Group (%)			[11C]-Group (%)		
	[12C]-short	[12C]-long		[11C]-short	[11C]-long	
B	33.3	72.8	(p<0.0001)	33.3	16.7	
B*	22.2	9.1		0	0	
Z	22.2	18.2		22.2	66.7	(p<0.0001)
M-a	22.2	0		22.2	0	
Others	0	0		11.1	16.7	
F	0	0		11.1	0	

One-base-pair insertion and deletion are very frequent in homonucleotide runs, which can be explained precisely by transient misalignment (see Rogozin et al. 2001; Ripley 1982). How the haplogroups B and Z influence the extent of this transient misalignment remains to be investigated.

Nuclear Factor(s) Involvement in Length Heteroplasmy Generation

In one of the families with the 16189C studied, length heteroplasmy was observed in certain maternally related members of the family only (Table 6); most (4/6) of the members examined did not show the length heteroplasmy, while in the rest (2/6) the pattern of the length heteroplasmy is relatively simple, consisting of two variants of eight and nine cytosines (Fig. 4.10). DNA sequencing revealed that this particular family carries a C16192T transition, interrupting the homopolymeric cytosines near the end of the tract, resulting in a homopolymeric tract of eight cytosines only. The 16192T/C variants had been studied in some details, and are frequently found in a heteroplasmic manner (Bendall et al. 1996; Howell and Smejkal 2000), suggested to be due to a very high reversion rate (hypermutation) of the 16192T in the presence of 16189C, preventing the rapid fixation toward homoplasmy typical of mtDNA. The 16192T/C heteroplasmy in the pedigree examined could not be detected, although the generation of both the tract length heteroplasmy and the hypermutation have been proposed to be due to a shared mechanism of replication slippage (Howell and Smejkal 2000).

Shorter homopolymeric tracts of cytosines have been reported to be more stable (Bendall and Sykes 1995; Marchington et al. 1996; Howell and Smejkal 2000). The observation that some maternally related members of family ElPw-97 show length heteroplasmy in their mtDNA while others do not is of interest in term of the factor that determine the generation of the length heteroplasmy, which in this case appears to be nuclear. A study to obtain a definitive evidence for the involvement of a nuclear factor in this process has been carried out. For this purpose, two fibroblast cell lines derived from members of this family have been employed. One cell line, ElPwII-06-97, was derived from a member carrying a single mtDNA species with eight poly [C] length (designated II-06[II-06]). The other, ElPwIII-10-97, was derived from a member which exhibits the heteroplasmic presence of two variants of the poly [C] with the ratio of 70% of eight and 30% of nine poly [C]s (III-10[III-10]; Fig. 4.7).

Table 4.6. Nucleotide Changes Near the 16189C Variant

Haplogroup	16055	16086	16092	16093	16129	16136	16140	16148	16154	16172	16179	16182	16183	**16189**	16199	16204	16213	16216	16217	16223	16226	16235	16247	16261	16266	16272	16274	16284	16297	16300	16304	16309	16310	16311	16319	16326	16335	16338	16342	16343	16354	16357	16358	16360	16362	16422	16430
CRS	C	T	T	T	G	T	T	C	T	T	C	A	A	**T**	T	G	G	A	T	C	A	A	A	C	C	T	G	A	T	A	T	A	G	T	G	A	A	A	T	A	C	T	C	C	T	T	A
AACCCCCCCCCCCC ([12C]-group)																																															
[12C]-short																																															
B*	.	.	.	.	.	.	.	.	.	.	.	C	C	C	.	.	.	.	C	T	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Z	.	.	.	.	.	.	C	.	.	.	.	C	C	C	.	.	.	.	.	.	.	.	.	T	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B	.	.	.	.	.	.	C	.	.	.	.	C	C	C	.	.	.	.	C	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	G	.	.	G	.	.	.	.	.	.	.
M	.	.	.	.	.	.	.	.	.	.	.	C	C	C	.	.	.	.	.	T	.	.	.	T	.	C	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.
Z	.	.	.	.	.	.	.	.	.	.	.	C	C	C	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B	.	.	.	.	.	.	C	.	.	.	.	C	C	C	.	.	.	.	C	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B	.	.	.	.	.	.	C	.	.	.	.	C	C	C	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.
B*	.	.	.	.	.	.	C	.	.	.	.	C	C	C	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	C	.	C	.	.	.	.	.	.	.	.	.
M	.	.	.	.	.	.	.	.	.	.	.	C	C	C	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.
[12C]-long																																															
B	.	.	.	.	.	.	.	.	.	.	.	C	C	C	.	.	.	.	C	.	.	.	G	T	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

```
B       . . . . . . . . . . . C C C . . . . C . . G . . . . . . . . . . . . . . . . . . . . . . . . .
B       . . . C . C . . . . T C C C . . . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . .
B       . . . . . . C . . . . C C C . . . . C . . . . T . . . . . . . . . . . . . . . . . . . . . . .
B       . . . . . . . . . . . C C C . . . . C . . . . . . . A . . . . . . . . . G . . . . . . . . . .
B       . . . . . . . . . . . C C C . . . . C . . . G T . . . . . . . . . . . . . . . . . . . . . . .
B       . . . . . . C . . . . C C C A A . . C . . . . . . . A . . . . . . . . . G . . . . . . . . . .
Z       . . . . . . C . . . . C C C . . . . . . . . . T A . . . . . . . . . . . . . . . . . . . . . .
B       . . . C . . . . . . . C C C . . . . C . . . G T . . . . . . . . . . . . . . . . . . . . . . .
Z       . . . . . . C . . . . C C C . . . . . . . . . T A . . . . . . . . . . . . . . . . . . . . . .
B*      . . . . . . C . . . . C C C . . . C . . . . . . . . . . . . . . . . C C . C A . . . T G . . C
```

AAACCCCCCCCCCC ([11C]-group)

[11C]-short

```
Others  . . . . . . . . . C . . C C . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Z       . . . . . . C . . . . . C C . . . . . . . . . . A . . . . . . . . . . . . . . . . . . . . . .
F       . . . . . . . . . . . . C C . . . . . . . . . . . . . . . G G . . . . . . . . . . . . . . . .
Z       . . . . . . C . . . . . C C . . . . . . . . . . A . . . . . . . . . . . . . . . . . . . . . .
B       . . . . . . . T G . . . C C . . . . . T . . . . . . . . . . . . . . . . G . . . . . . . . C .
B       . . . C . . C . . . . . C C . . . . C . . . . . . . A . . . G . T . . . G . . . . . . . . . .
M       . C . . A . . . . . . . C C . . . . . T . . . . . . . . C . . . . . . . . . . . . . . . . . .
B       . . . . . C . . . . . . C C . . . . C . . . . . . . . . . . . C . . . . . . . . T . . . . . .
```

M	. . C . . . . T . . G . C C . . A . . T . . . . . C . . . . C . . . .
[11C]-long	
B	. . C . . . . T . . . . C C . . . . C . .
Z	T . . . A . C . . . . . C C . . . . A . . . .
Z	. . . . C C . . . . A . . . .
Z	. . . . . . C . G . T . C C . . . . A . . . .
Others	. . . . C C . . . . . T . . . . C . . C . .
Z	. . . . . . C . . . . . C C . . . . A . . . . C .

CRS = Cambridge reference sequence (Anderson et al. 1981). [12C]-short = mean length of the poly [C] of 12-12.5; [12C]-long = mean length of the poly [C] of >12.5; [11C]-short = mean length of the poly [C] of 11.2-12; [11C]-long = mean length of the poly [C] of >12.

Figure 4.10. Length Heteroplasmy in A Family with the 16189C and 16192T variants.

Maternally related members (∇) of family ElPw-97 carrying the 16189C and 16192T variants (upper panel) were examined for the pattern of the length heteroplasmy as described in Fig. 4.1. The relative proportions of the length variants associated with the 16189C in the family members examined are shown under the pedigree.

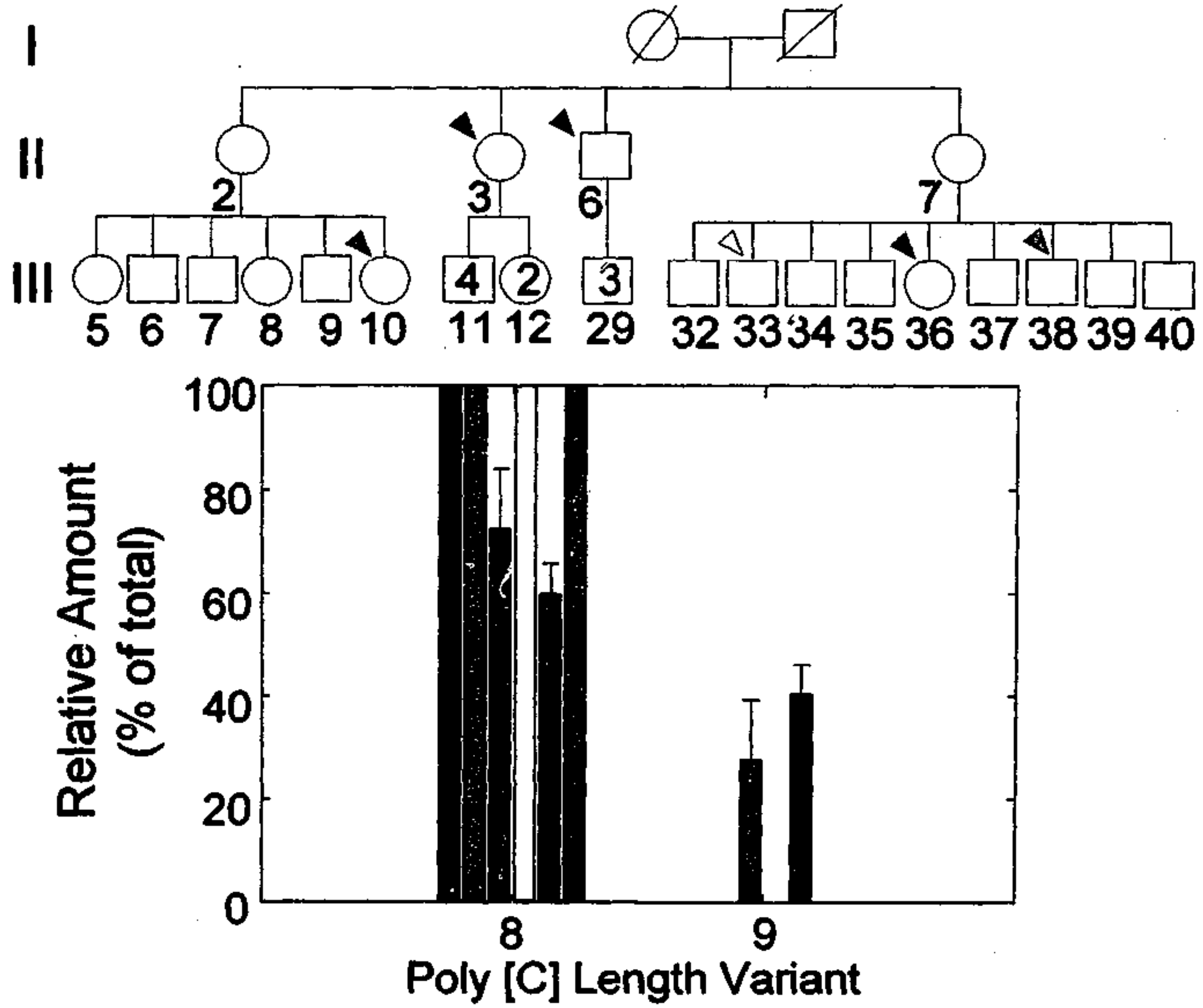

I
II
III
2
3
6
7
5
6
7
8
9
10
4
2
11
12
3
29
32
33
34
35
36
37
38
39
40
100
80
60
40
20
0
Relative Amount
(% of total)
8
9
Poly [C] Length Variant

Table 4.7. Relative Proportions of the Poly [C] Length Heteroplasmy in Members of Family ElPw-97 Carrying the 16189C and 16192T Variants

Family member	Poly [C] length heteroplasmy	
	8 C (%)	9 C (%)
II-03	100	
II-06	100	
III-10	72	27
III-33	100	
III-36	60	40
III-38	100	

Cybrid cells were constructed from the two fibroblast cell lines above to investigate whether the length heteroplasmy associated with the mtDNA of EIPwII-06-97 and EIPwIII-10-97 changes when their respective nuclear background is switched. In the first set of experiments, a mtDNA-less ρ^0 cell line (EIPwIII-10[ρ^0]) was generated from EIPwIII-10-97 (carrying β-globin gene polymorphic sites as nuclear markers [β-C/β-C]) by exposure to ethidium bromide (Fig. 4.2). As the mtDNA donor, the parental EIPwII-06-97 cells (carrying only one length variant of eight poly [C] in the mtDNA) were enucleated (King and Attardi 1996) and fused with the mtDNA-less EIPwIII-10[ρ^0] cells (which originally have two variants of eight and nine poly [C] in the mtDNA). In all seven cybrids examined (designated III-10[II-06]), the mtDNA was found to be of two species, the eight and nine poly [C] variants (Fig. 4.11).

The experiment was carried out in reverse by introducing the nucleus of mtDNA-less EIPwII-06-97 cells (originally carry only one morph of eight poly [C] in the mtDNA) into the enucleated EIPwIII-10-97 cells (with two morphs of eight and nine poly[C]s in the mtDNA). In this case, the mtDNA of the donor EIPwIII-10-97 cells, which is now in the background of EIPwII-06-ρ^0 nucleus showed only one morph instead of the original two morphs (Fig. 4.12), confirming that the length heteroplasmy generation in this case is a nuclearly determined characteristic.

Figure 4.11. Cybrids Construction Shows that the Generation of Length Heteroplasmy in the Homopolymeric Tract of Eight Cytosines is Nuclearly Determined (1).

Shown are the patterns of the length heteroplasmy of the parental cells, II-06[II-06] and III-10[II-10], and in two cybrids, III-10[II-06]#07 and III-10[II-06]#09 showing the presence of length heteroplasmy.

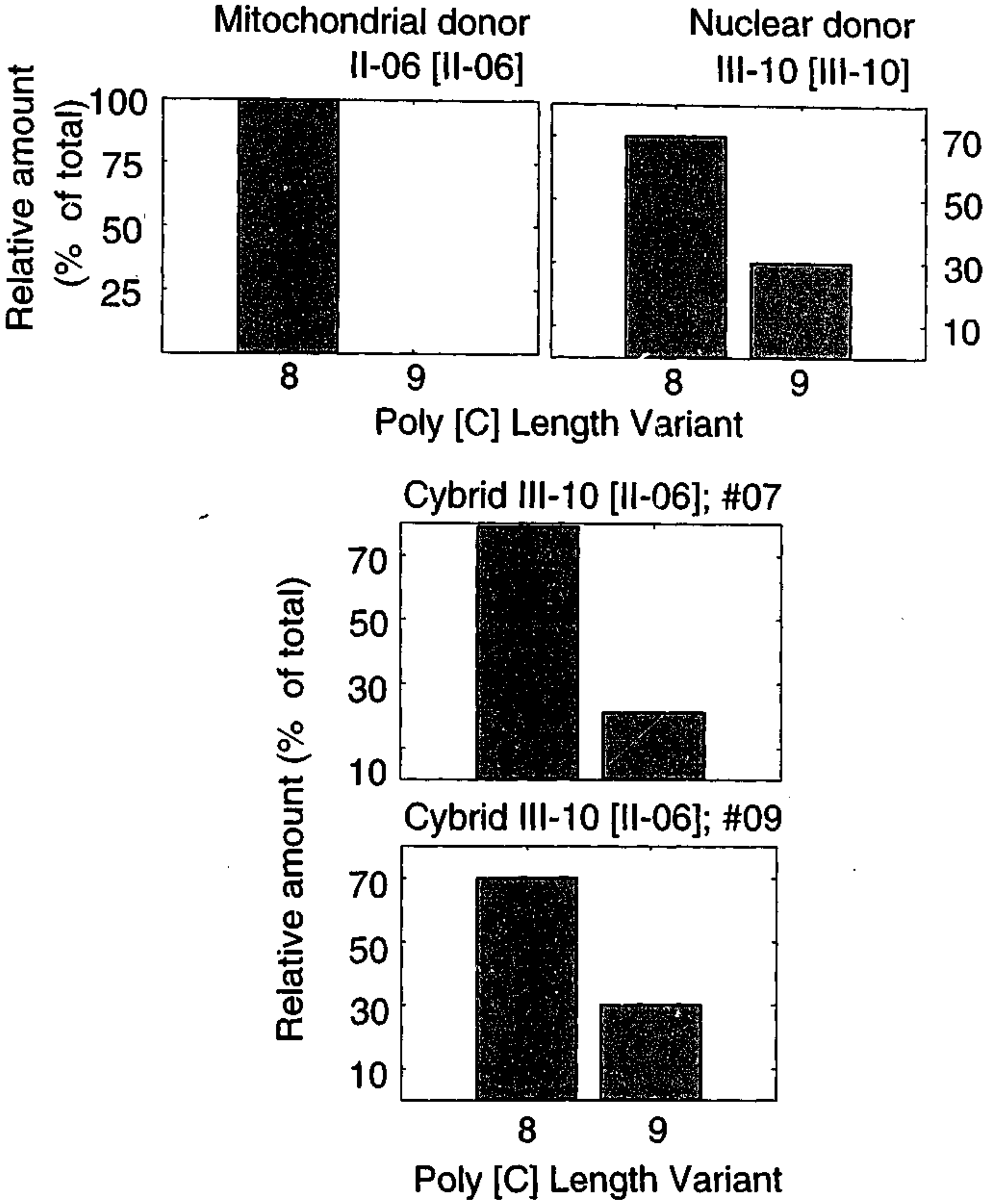
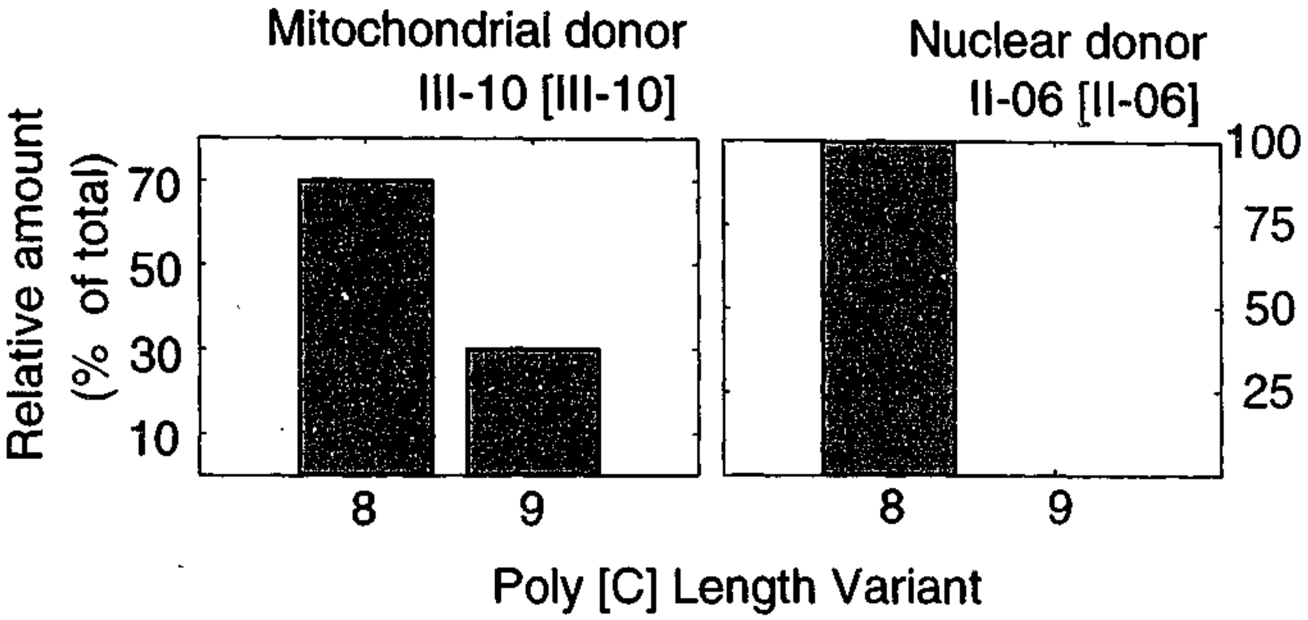
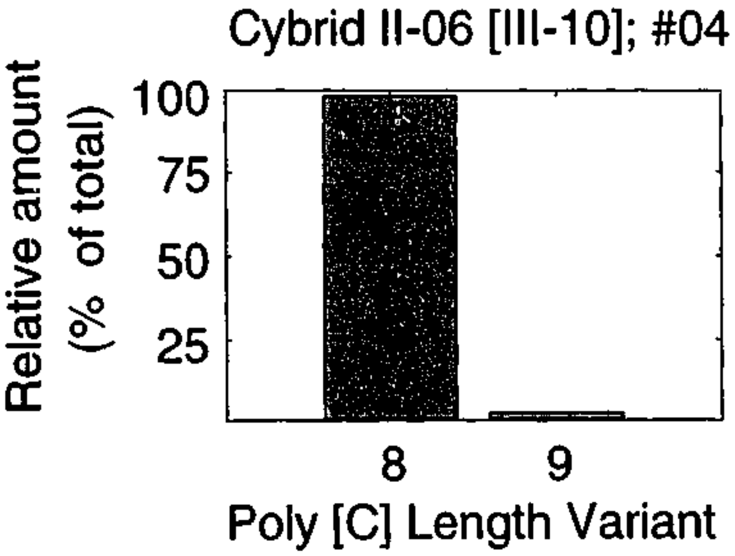

Mitochondrial donor
II-06 [II-06]
Nuclear donor
III-10 [III-10]
Relative amount
(% of total)
100
75
50
25
70
50
30
10
8
9
8
9
Poly [C] Length Variant
Cybrid III-10 [II-06]; #07
Cybrid III-10 [II-06]; #09
Relative amount (% of total)
70
50
30
10
70
50
30
10
8
9
Poly [C] Length Variant

Fig. 4.12. Cybrids Construction Shows that the Generation of Length Heteroplasmy in the Homopolymeric Tract of Eight Cytosines is Nuclearly Determined (2).

Shown are the patterns of the length heteroplasmy of the parental cells, II-06[II-06] and III-10[II-10], and in a cybrid, II-06[III-10]#04 showing the presence of a major eight-cytosine morph and a much less nine-cytosine morph.


Mitochondrial donor
III-10 [III-10]
Nuclear donor
II-06 [II-06]
Relative amount
(% of total)
70
50
30
10
100
75
50
25
8
9
8
9
Poly [C] Length Variant


Cybrid II-06 [III-10]; #04
Relative amount
(% of total)
100
75
50
25
8
9
Poly [C] Length Variant

The nuclear factor(s) responsible for the generation of the length heteroplasmy is/are most likely to be associated with the fidelity of the mtDNA replication machinery, and could be any one of the components involved in the mtDNA replication system, such as sequence polymorphisms in the DNA polymerase γ. It has been reported that the 140 kDa catalytic subunit of the DNA polymerase γ (Ropp and Copeland 1996) harbours two activities; the 5'→3' polymerase/processivity activity at the COOH-terminal of the enzyme (1996; Foury 1989; Ropp and Copeland 1995), and the 3'→5' exonucleolytic activity involved in proofreading at its NH_2-terminal (1996; Ropp and Copeland 1995; Graves et al. 1998; Longley et al. 1998). The exonucleolytic proofreading activity contributes to frameshift fidelity during the replication of repetitive DNA sequences, but the contribution reduces and diminishes as the number of repeats in the target sequence increases (Kunkel et al. 1994). In the case of the eight cytosine homopolymeric tract, the length heteroplasmy generation in some members of the ElPw-97 family could be due to the variation in the 3'→5' exonuclease proofreading activity.

It has been attempted to identify the nuclear factor(s) involved in the generation of the length heteroplasmy by studying the 5' end of the DNA polymerase γ gene, from nt362 to nt526. The NH_2-terminal of the catalytic subunit of the human DNA polymerase γ contains a stretch of poly-glutamine coded by CAG repeats within its coding region (Ropp and Copeland 1996; Zullo et al. 1997; Lecrenier et al. 1997). The predominant allele in the human population contains 10 CAG repeats, followed by a single CAA and two further CAGs, thus coding for a stretch of 13 glutamines. Rare polymorphisms have been reported resulting from a variation in the first stretch of CAG between 5-13 repeats (Rovio et al. 1999). The sequencing data obtained did not show any variation of the repeats; all six members of the family studied have 10 CAG repeats. This rare polymorphism, therefore, is not informative for the investigation of a possible linkage of the variation in the mtDNA replication fidelity with DNA polymerase γ.

CONCLUDING REMARKS

This chapter has presented evidence demonstrating that the length heteroplasmy associated with homopolymeric tracts in the mtDNA is generated under the influence of both nuclear and mitochondrial factors. The mtDNA replication machinery appears to be able to replicate the homopolymeric tract faithfully up to the length of six cytosines; in a general survey of homopolymeric tracts in the mtDNA, all three stretches of six cytosines found do not show length variation (Table 4.8). At poly [C] length of seven (Table 4.8) or eight (the present study) the length heteroplasmy could be observed but only in certain individuals, indicating the existence of population variants in the fidelity of the nuclearly coded mtDNA replication machinery. The presence of such a nuclearly coded determinant is experimentally demonstrated in the present study, but the exact molecular basis remains to be investigated. DNA polymerases, including the DNA polymerase γ, act in cooperation with and depend on a number of additional proteins to accomplish a faithful genomic replication. Sequence polymorphisms in one or more of the genes coding for these proteins, such as the 55 kDa accessory subunit (Lim et al. 1999) that improves the fidelity of DNA synthesis, or the mitochondrial single-stranded DNA binding (mtSSB) protein that has been reported to improve the proofreading capability by enhancing the mismatch-specific 3'→5' exonucleolytic activity (Gray and Wong 1992) are also potential candidates for the nuclear factor(s) that could enhance or reduce the fidelity of the DNA polymerase γ.

At the homopolymeric tract lengths of longer than eight cytosines, the fidelity of the mtDNA replication machinery appears to decline significantly, generating length heteroplasmy in all individuals carrying the tracts. In this case, the generation of the length heteroplasmy is obviously influenced by mitochondrial factors, as maternally related members of a family always show a similar pattern of length heteroplasmy. The mitochondrial factor(s) is/are most likely to be the mtDNA sequence polymorphisms that form a background against which the length heteroplasmy associated with a homopolymeric tract is generated. It has been shown that the pattern of the length heteroplasmy with the modal length of 12Cs (^{16180}AACCCCCCCCCCCC^{16193}) is associated with haplogroup B, whereas those with the modal length of 11 Cs (^{16180}AAACCCCCCCCCCC^{16193}) with haplogroup Z, but attempts to define the exact nucleotides involved have not been successful. While cannot be mechanistically explained as yet, the result obtained in this chapter seems to indicate that the mitochondrial determinants include multiple sequence variants in the mtDNA, which synergistically interact in influencing the mtDNA replication fidelity.

Table 4.8. Length Heteroplasmy Associated with Homopolymeric Cytosine Tracts in the mtDNA

Homopolymeric tract	Associated length morphs	Length of poly [C]	Length morphs
$^{71}\text{CCCCCC}^{66}$	none	6C	none
$^{303}\text{CCCCCCCTCCCCC}^{315}$	none	7C(+5C)	7-10C
	T310C	13C	13-16C
$^{16180}\text{AAAACCCCCTCCCC}^{16193}$	none	5C(+4C)	none
	A16183C	6C	none
	T16189C	10C	8-14C
	A16183C+T16189C	11C	8-15C
	A16182C+A16183C+T16189C	12C	8-15C
$^{16375}\text{CCCCCC}^{16380}$	none	6C	none

APPENDICES

APPENDIX 1

THE PRODUCTION OF ANTIPEPTIDE ANTIBODIES SPECIFIC TO SUBUNITS OF THE HUMAN NADH-COQ OXIDOREDUCTASE

Epitope specific antibodies against subunits of the respiratory enzyme complexes have been part of the powerful array of tools employed in the study of the biosynthesis and assembly of these enzyme complexes as well as their defects. Such antibodies are useful, for example, as immunoprobes for tissue and cell localization (Collins et al. 1991), in assays of the enzyme assembly defect (Hadikusumo et al. 1988; Noer et al. 1992), and as probes during enzyme purification process (Hadikusumo et al. 1986; Jean-Francois et al. 1988). Antibodies with a predetermined specificity, raised against short peptides, which corresponds to parts of the native proteins, have been successfully used for this purpose. Such antibodies were needed for the study described in Chapter 2 and thus have been raised as part of my PhD work.

Antibodies against four subunits of NADH-CoQ oxidoreductase: two of the mitochondrial coded subunits (ND1 and ND4), and two of the nuclear coded subunits (75 kDa NDUFS1 subunit and 24 kDa NDUFV2), were raised. The peptide sequence corresponding to the carboxy terminal regions of the four subunits are shown in Table A1.1. The peptide design corresponding to the ND1 and ND4 subunits of NADH-CoQ oxidoreductase are as previously described (Chomyn et al. 1986). The amino acid sequence of the other two peptides were chosen based on the antigenic index, immunogenicity, hydrophilicity, and hydropathy index corresponding to the carboxy terminal region of the nuclearly coded subunits of the published sequence of the NDUFS1 subunit of the IP fraction (Ragan *et al*, 1991) and the NDUFV2 subunit of the FP fraction (Pilkington and Walker, 1989). An extra lysine residue was added to each peptide at their amino terminal end, except for the peptide corresponding to the to facilitate the conjugation. All peptides were commercially synthesized (Chiron Mimotopes, Melbourne, Australia). Coupling of the synthetic peptides with either keyhole limpet haemocyanin (KLH; Calbiochem, USA) as an immunogenic carrier were performed by using 2.5 % glutaraldehyde following a modification of a two-step method (Sattayasai et al. 1991).

Rabbits (in duplicates) were immunized intramuscularly with KLH-conjugated peptides (0.75-1 mg of peptide per rabbit), emulsified in Freund's complete adjuvant (total volume 1 ml per rabbit), for the primary injection. Booster injections were carried out at two weekly intervals after the primary injection, using the same amount of peptide in incomplete Freund's adjuvant. Rabbit sera was collected one week after each injection, and anti-peptide antibodies against the peptides in the sera were screened by direct Enzyme-Linked Immunosorbent Assay (ELISA) with the peptides coupled to a protein unrelated to the immunizing carrier. In all cases the antisera did not show any reactivity to BSA for peptides conjugated with KLH, or to KLH for peptide conjugated with BSA. The antibodies against each peptide in the immune sera reached the maximum titre of between 1:10,000 to 1:45,000 after four booster injections (data not shown).

The reactivity of the antipeptide antibodies to subunits of the NADH-CoQ oxidoreductase by Western immunoblotting against the human placental mitochondrial protein. One band of corresponding molecular weight was observed, as represented in Fig. A1.1

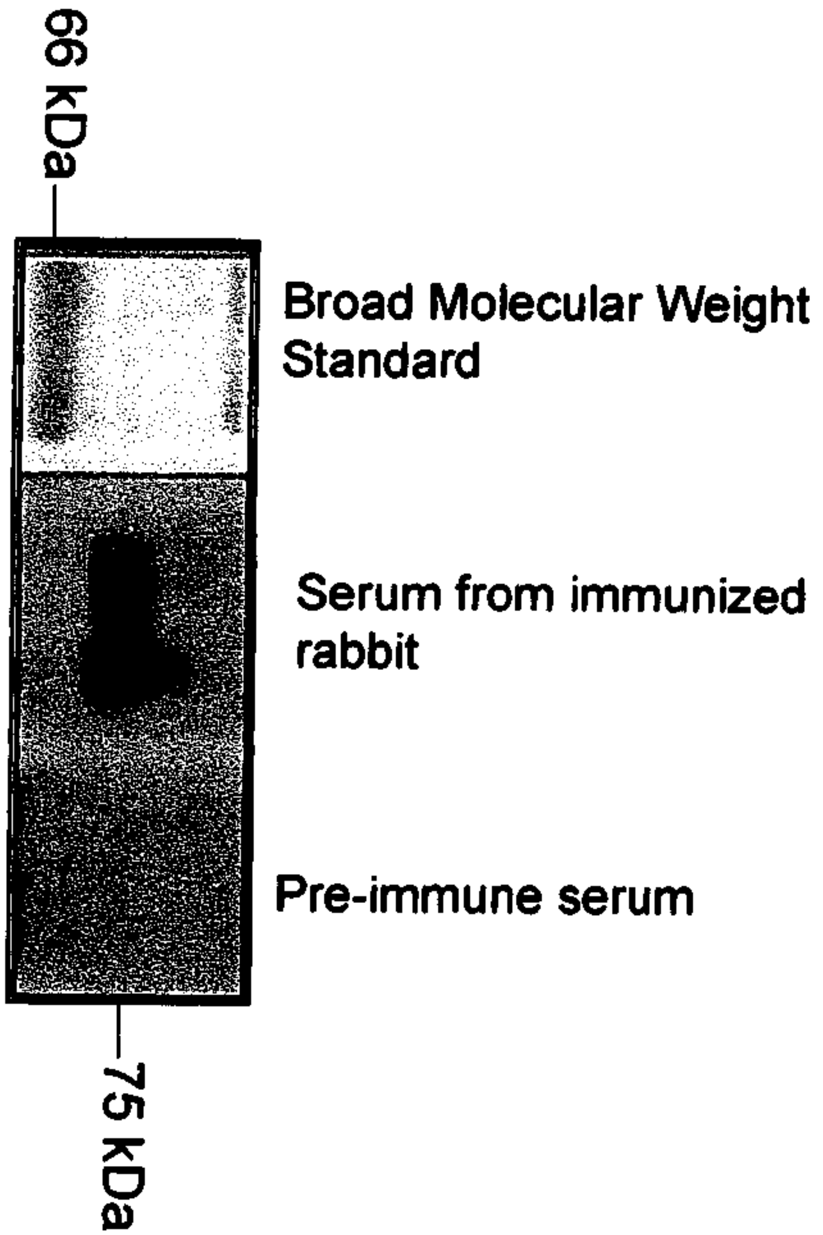
Table A1.1. Synthetic Peptides Used in the Production of Epitope Specific Antibodies to Subunits of NADH-CoQ Oxidoreductase

Sequence of Peptide	Position (Amino acid residue)	Region	NADH-CoQ oxidoreductase Subunit
[NH3]-*K*-P-I-T-I-S-S-I-P-P-Q-T-[COOH]*	308-318	C-terminal	ND1
[NH3]-*K*-P-D-I-I-T-G-F-S-S-[COOH]*	451-459	C-terminal	ND4
[NH3]-*K*-E-G-A-Q-A-V-E-E-P-S-I-[COOH]	716-726	C-terminal	NDUFS1**
[NH3]-K-G-P-G-F-G-V-Q-A-G-L-[COOH]	239-249	C-terminal	NDUFV2***

K (lysin) is added and not from the original sequence of the polypeptide. *Amino acid sequences are as described previously [Chomyn *et al*, 1985]. **Accession number is P28331 [Ragan *et al*, 1991]; ***accession number is P19404 [Pilkington and Walker, 1989].

Fig A1.1. The Specific Reactivity of the Epitope Specific Antibody to the NADH-CoQ oxidoreductase subunits in Western Immunoblotting

Human placental mitochondrial protein (18 µg/µl) were separated by electrophoresis in 12.5% SDS-polyacrylamide gel, electrophoretically transferred onto nitrocellulose filter, and immunoblotted with antipeptide antibodies against the NDUFSI subunit of the NADH-CoQ oxidoreductase. A band corresponding to a 75 kDa protein is demonstrated.


66 kDa
Broad Molecular Weight Standard
Serum from immunized rabbit
Pre-immune serum
75 kDa

APPENDIX 2

VERIFICATION OF A NOVEL METHOD FOR QUANTITATION OF THE HOMOPOLYMERIC TRACT LENGTH HETEROPLASMY

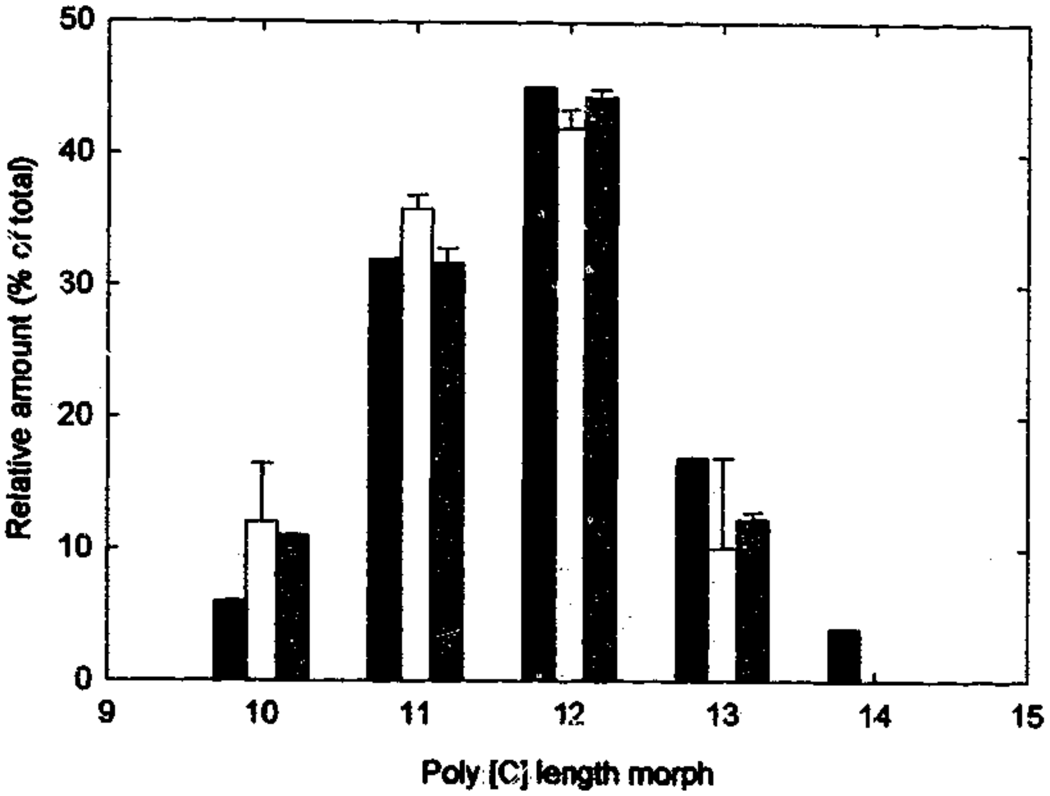
The D-loop region of the mtDNA contains homopolymeric tracts that exhibit length polymorphisms. Of particular interest is nt 16189T, located within and interrupts a homopolymeric tract of cytosines between nt16184 and nt16193. A T16189C mutation occurs in some individuals resulting in an unstable homopolymeric tract of ten cytosines, and length variants of between 8 to 14 cytosines of the poly [C] is observed. To study the proportion of the length heteroplasmy described in Chapter 4 Marchington et al (1996) has developed a T-PCR (for trimmed PCR) to analyze the homopolymeric tract length variation. PCR products are immobilized by binding the downstream biotinylated primer to Streptavidin coated magnetic beads, leaving the upstream primer adjacent to the homopolymeric tract exposed, producing single stranded products for electrophoretic analysis. A novel relatively simpler procedure was developed to quantitate the poly [C] length polymorphism described, based on PCR amplification of a 174 bp fragment spanning from nt16142 to 16313 of the mtDNA (the poly [C] in the Cambridge reference sequence is from nt16184 to 16193). A restriction endonuclease (*Rsa*I), which produces blunt end products, is employed to trim the 5' and 3' end of the PCR products, resulting in poly [C] containing fragments of 50-56 bp, depending on the length of the poly [C] (artifactual length variations due to a 1-bp overhang at the 3' end introduced by *Taq* polymerase would otherwise complicate the analysis of the length of the poly [C]). The relative proportions of the various morphs are quantitated after the separation of the trimmed PCR products in 20% polyacrylamide gel (run at 2300 V for 4 hours), and visualization with either silver staining or autoradiography of [^{33}P]-labeled products. The use of a high concentration of polyacrylamide gel is due to the difference of the electrophoretic property of heavy- and light- strand of the mtDNA, where the heavy strand run slower than the light strand, and it will complicate the analysis of the length heteroplasmy proportion.

To ensure that the quantitative method developed is reliable, a 410 bp PCR products spanning from nt15904 to 16313 containing the poly [C] tract were cloned into M13, since each clone only contain one mtDNA molecule, and cloning is the gold standard to determine the proportion of heteroplasmic mtDNA. Seventy five of these clones were sequenced, and we found that the proportion of the poly [C] tract length was identical (Fig. A2.1).

The result showed that the novel method developed is reliable, quick and simple to be employed as a method to quantitate a homopolymeric tract polymorphism.

Fig A2.1. Quantitation of the poly [C] length morphs.

The pattern of length heteroplasmy determined by sequencing 75 of the clones mentioned in the text (black), or quantitated after visualization with either silver staining (white) or autoradiography of [33P]-labeled products (red). The pattern determined by quantitation of the silver stained or autoradiographed acrylamide gels are the results of at least three independent duplicate measurements.


50
40
30
20
10
0
Relative amount (% of total)
9
10
11
12
13
14
15
Poly [C] length morph

APPENDIX 3

THE CONSTRUCTION OF MITOCHONDRIAL AND NUCLEAR DNA INTERNAL STANDARDS

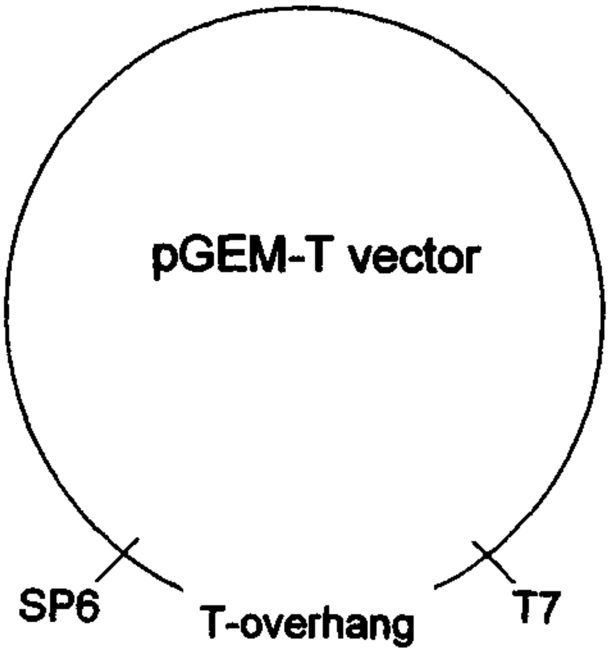
For the mtDNA and nuclear DNA quantitation in Chapter 4, internal standards for both DNA were needed. These standards have been constructed as part of the development of a reliable quantitative PCR method in our laboratory to analyze the amount of mtDNA in human tissues.

The internal standard for the mtDNA is a 1673 bp mtDNA fragment spanning nt2055 – nt3728. The primer pair employed to amplify the insert is the modified L2055 (5'-TGCCCACAGAACCCTCTAAATCCCCTTGTAAATTTAACTGTTAGTCCAAAGAGGAACAGCGCCTTGGAGGCTAGG-3'), which introduced the *BglI* restriction site (underlined) that is not present in the normal mtDNA to allow the differentiation of the PCR products of the internal standard from the mtDNA being examined, and H3728 (5'-GAGATTGTTTGGGCTACTGCTCGC-3'). The amplified product was then inserted into the pGEMT-easy vector (Promega, USA; Fig. A3.1a). Quantitative PCR was performed by employing primer pair L2055 (5'-TGCCCACAGAACCCTCTAAATCC-3') and H2343 (5'-CAGGCTTATGCGGAGGAGAATGTT-3'), resulting in 289 bp fragment. Digestion of the 289 bp PCR-amplified insert by *BglI* restriction endonuclease (Gibco, BRL, USA) resulted in 222 and 67 bp fragments, respectively (Fig. A3.1b).

The internal standard for the nuclear DNA is a 123 bp PCR-amplified insert of the α1-globin gene, which has lost the *HinfI* restriction site at codon 116 (Fig. A3.2a). The primer pair used to amplify the 123 bp insert is the ALF1 (5'-CGGCTGCGGGCCTGGGCCCTCGGCCC-3') and HbO (5'-AGGCGT GCACCGCAGGGGTGGAC-3'). Another internal nuclear DNA standard was constructed with an insert of the same 123 bp PCR-fragment, but with the *HinfI* restriction site at codon 116 present. This second internal standard is used as control for *HinfI* digestion, resulting in 98 and 25 bp fragments (Fig. A3.2b).

Fig. A3.1. Diagram of the mtDNA Internal Standard.

The internal standard for the mtDNA is a 602 bp mtDNA fragment spanning nt2055 – nt3728 which has been inserted into the pGEMT-easy vector, carrying a PCR-introduced *BglI* restriction site, which is not present in the normal mtDNA, allowing the differentiation of the PCR products of the internal standard from the mtDNA being examined.


pGEM-T vector
SP6
T-overhang
T7

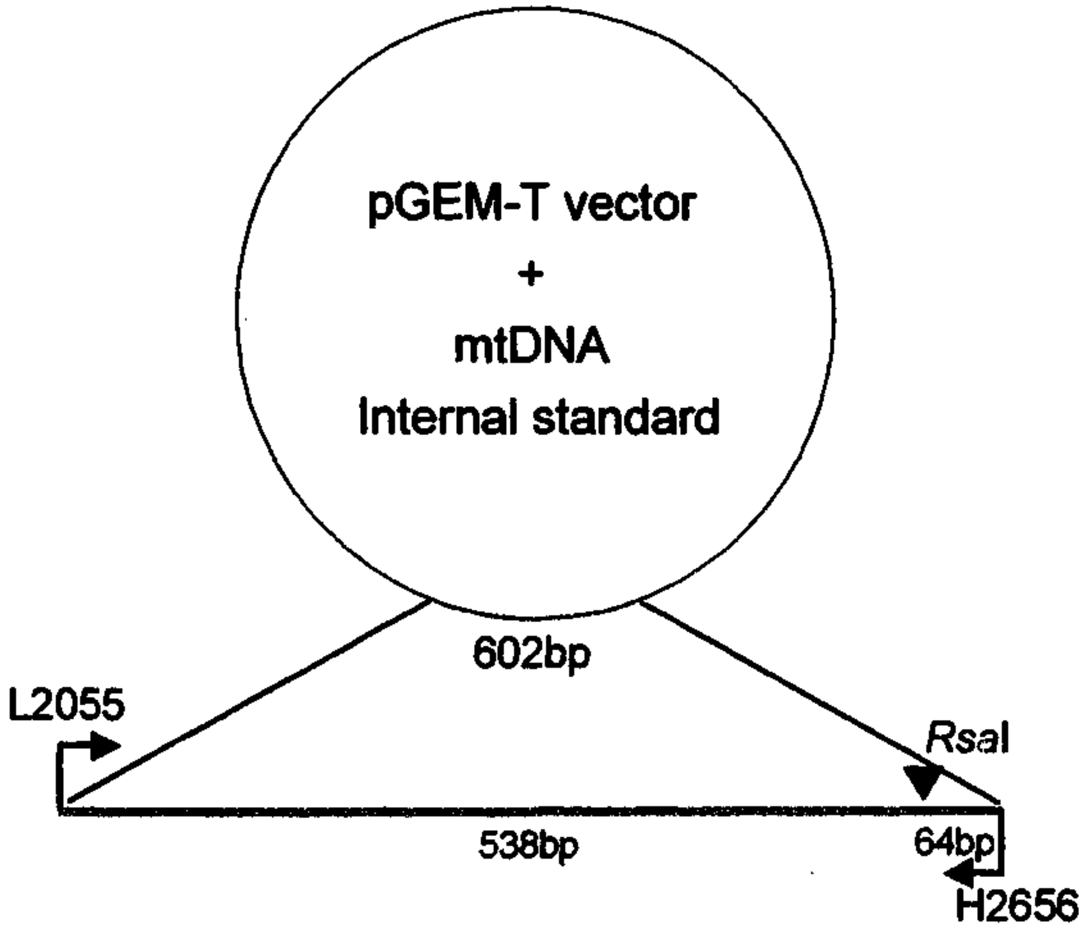
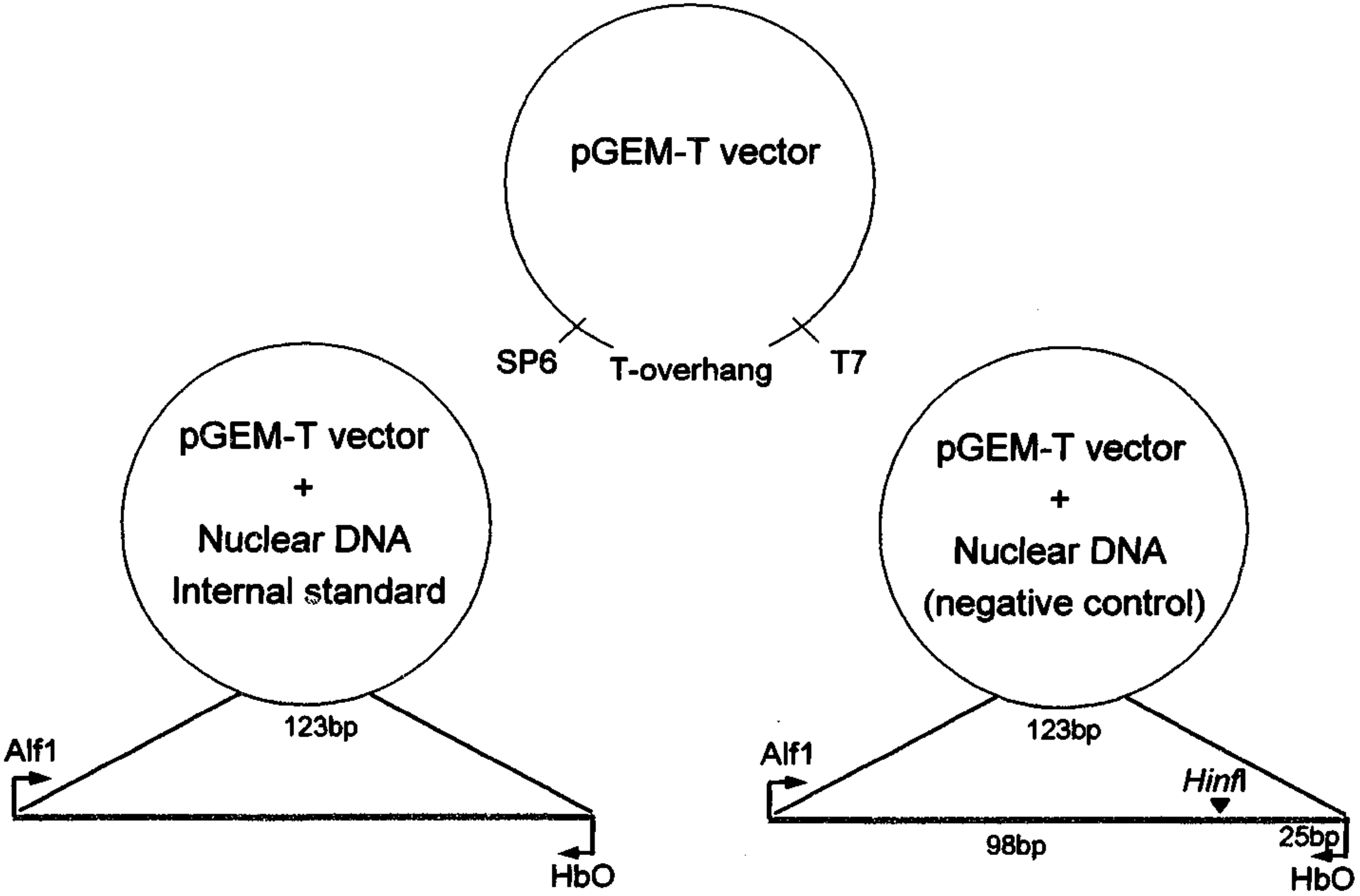

pGEM-T vector
+
mtDNA
Internal standard
602bp
L2055
RsaI
538bp
64bp
H2656

Fig. A3.2. Diagram of the Nuclear Internal Standard.

The internal standard for the nuclear DNA is a pGEMT-easy vector with a 123 bp PCR amplified insert of the α1-globin gene, which has a rare mutation causing a lost of a *Hinfl* restriction site at codon 116. This mutation is only found in individuals with Haemoglobin O Indonesia (HbO_{Ina}), restricted to the populations of South Sulawesi.


pGEM-T vector
SP6
T-overhang
T7
pGEM-T vector
+
Nuclear DNA
Internal standard
123bp
Alf1
HbO
pGEM-T vector
+
Nuclear DNA
(negative control)
123bp
Alf1
HinfI
98bp
25bp
HbO

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