The role of mitochondrial mutations
in sensorineural hearing loss

by

Fiona M Reid, BSc, MSc

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Institute of Biomedical and Life Sciences,
Robertson Laboratory of Biotechnology

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

More things are wrought by prayer than this world dreams of.

Idylls of the King
by Alfred, Lord Tennyson

I wish to dedicate this thesis to the memory
of my Mother and of my Uncle Tommy

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The research reported in this thesis is my own original work, except where otherwise stated, and has not been submitted for any other degree.

SUMMARY

Sensorineural hearing loss is often the first symptom reported in a number of mitochondrial myopathies. Neuronal tissue is very sensitive to alterations in oxidative phosphorylation which often occur in these diseases. During an investigation of thirty three patients with non-syndromic sensorineural hearing loss by RFLP (Restriction Fragment Length Polymorphism) analysis, for known mutations associated with specific mitochondrial mutations, one patient was found to have lost the *Xba*I site at np 7440.

This patient is part of a large maternal pedigree in which 14 members suffer from some degree of sensorineural hearing loss. The entire mitochondrial DNA sequence of this patient was sequenced revealing that the *Xba*I site loss was due to a homoplasmic A->G change at np 7445. The nucleotide at this position has a role on both strands: part of the stop codon of the COI (Cytochrome Oxidase subunit I) gene on the major coding strand and the extreme 3' nucleotide of $tRNA_{ser(UCN)}$ on the other.

The biochemical effects of this mutation were investigated in permanent cell-lines created by EBV (Epstein Barr Virus) transformation of lymphocytes from the index case, and from a normal control. In studies of lactate/pyruvate ratios, Complex II + III activity, Complex IV activity, growth with galactose as the carbon source and in the presence of chloramphenicol there was no difference between the two cell-lines. Measurement of growth in the presence of various concentrations of streptomycin was carried out twice with inconclusive results.

In addition Northern blotting experiments indicated no difference in the level or length of the COI transcript in the cell-line with the np 7445 mutation. Mitochondrial translation products were measured by ^{35}S-labelling in the presence of emetine and no differences seen in the amount or sizes of any of the labelled proteins.

The entire mitochondrial DNA of a patient with LHON (Lebers Hereditary Optic Neuropathy) and sensorineural hearing loss was sequenced and found to have the so-called "classical" LHON mutation at np 11778.

In addition two patients with aminoglycoside antibiotic induced deafness were found to have a previously reported, homoplasmic A->G at np 1555 in 12SrRNA.

The work in this project has established that two mitochondrial DNA mutations are found in association with sensorineural hearing loss. That at np 1555 had previously been reported; that at np 7445 was novel. Both mutations are at positions of the human mitochondrial genome which have some direct involvement in mitochondrial translation.

LIST OF ABBREVIATIONS

AAID	Aminoglycoside Antibiotic Induced Deafness
ARMS	Amplification Refractory Mutation System
COX	Cytochrome Oxidase
CPEO	Chronic Progressive External Ophthalmoplegia
CSF	Cerebro Spinal Fluid
dB	Decibels
DIG	Digoxigenin
DIDMOAD	Diabetes Insipidis Diabetes Mellitis Optic Atrophy and Deafness
DM	Diabetes Mellitis
EBV	Epstein Barr Virus
ECACC	European Collection of Animal Cell Cultures
ENT	Ear Nose and Throat
KSS	Kearns Sayre Syndrome
LHON	Lebers HereditaryOptic Neuropathy
MELAS	Mitochondrial Encephalomyopathy Lactic Acidosis and Stroke-like episodes
MERRF	Myoclonic Epilepsy with Ragged Red Fibres
NARP	Neurogenic muscle weakness, Ataxia and Retinitis Pigmentosa
NRF	Nuclear Respiratory Factor
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
SCCR	Succinate Cytochrome c Reductase
SSCP	Single Stranded Conformational Polymorphism

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CHAPTER 1 INTRODUCTION

1:1 MITOCHONDRIA - A BRIEF REVIEW

Mitochondria are cytoplasmic organelles present in almost all eukaryotic cells in varying numbers; up to hundreds per cell. Each is enclosed by a double membrane, an outer membrane separating the mitochondrion from the cytosol and an inner membrane enfolded to form cristae, which partially divide the inner compartment. The two membranes are separated by an inter-membrane space and the area internal to the inner membrane is known as the matrix.

The mitochondrion is often referred to as "the powerhouse of the cell" and this description is apt as it is the site of oxidative phosphorylation in which most of the ATP used within the cell is produced. Approximately 100 polypeptides involved in this oxidative phosphorylation process are located in the inner mitochondrial membrane in an extremely ordered fashion: See Figure 1:1. There are multi subunit complexes which span the inner mitochondrial membrane, through which electrons are passed. The electrical potential drives protons out across the membrane at complexes I NADH-ubiquinone reductase, III cytochrome-*c* reductase and IV cytochrone-*c* oxidase until at complex V, i.e. ATP synthase, protons are brought back across the membrane into the matrix where they take part in the formation of ATP.

1:1:1 Mitochondrial DNA

Mitochondria have their own DNA, quite separate from the cell's nuclear DNA. Approximately 1% of mammalian cellular DNA is mitochondrial. The size, organisation and control of expression of mitochondrial DNA is diverse throughout eukaryotes: size ranging from 16 kb in mammals to 2400 kb in plants. In some eukaryotes there are large non-coding regions and introns within genes; a small number of essential functions have been preserved in a wide range of species. Animal mitochondrial DNA is much more compact than in most plants and lower eukaryotes, ranging in size from about 13 kb to about 20 kb, with the organisation of genes within the genome being relatively highly conserved.

Figure 1:2 shows the gene organisation of human mitochondrial DNA and is typical of animal mitochondrial DNA. The human mitochondrial genome is a circular, double-stranded DNA molecule of 16,569 base pairs which codes for the two mitochondrial ribosomal RNAs, 12S & 16S, the small ribosomal subunit (SSU) and the large ribosomal subunit (LSU) respectively; 22 tRNAs and the mRNAs coding for 13 of the subunits of the oxidative phosphorylation pathway.

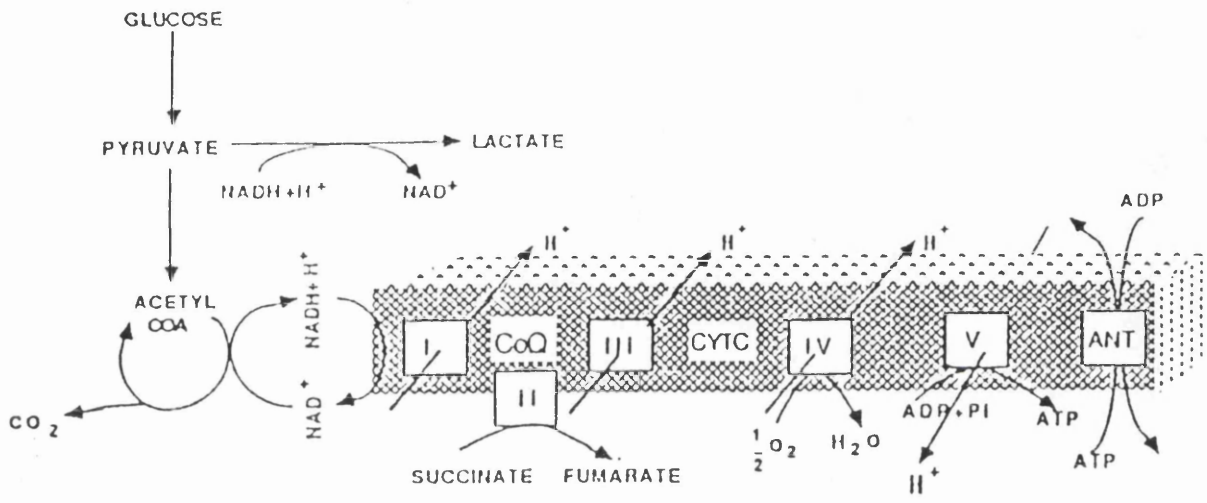


Figure 1:1 Mitochondrial Respiratory Complexes within the inner mitochondrial membrane.

CoQ - Coenzyme Q
CYTC - Cytochrome *c*
ANT - Adenine Nucleotide Transporter

Adapted from: Wallace et al, 1988.

Seven of these 13, ND1, ND2, ND3, ND4, ND4L, ND5 and ND6 are subunits of Complex I; cytochrome *b* is part of Complex III; COI, COII and COIII are three of the 13 subunits of Complex IV and ATPase 6 and 8 are subunits of Complex V. 22 tRNAs are sufficient for translation of the protein coding sequences of the human mitochondrial genome, due to a more simplified codon-anticodon pairing than is required to read the nuclear genetic code. The number of tRNAs varies slightly between species, but in humans eight mitochondrial tRNAs recognise 8 codon families with 4-fold degeneracy and, 14 recognise the remaining codon pairs (Shoffner and Wallace 1990).

The two strands of mitochondrial DNA can be separated according to density difference due to a strand bias in GT content. The so-called heavy strand (H) has the greatest density and encodes more genes than the less dense light (L) strand.

There are only two short non-coding regions in mammalian mitochondrial DNA: at the origin of light strand replication (O_L) and the D-loop. The D-loop is the least conserved region of the mitochondrial DNA, containing the predominant control regions for both replication and transcription: the origin of H strand replication (O_H) and both H and L strand promoters.

The H-strand of animal mitochondrial DNA begins replicating before the L-strand. The origin of L-strand replication is approximately 2/3 of way round the genome (clockwise on Figure 1:2) from the origin of H-strand replication. H-strand replication starts at O_H using the L-strand as template. The D-loop is formed by displacement of the parental H-strand from the L-strand by short H-strand sequences which function in priming DNA synthesis. Initiation of L-strand synthesis occurs when the growing H-strand exposes the single stranded parental H-strand which can be used as template for synthesis of the L-strand. L-strand synthesis then continues, counter clockwise as shown in Figure 1:2 (Nelson, 1987).

The H and L strand promoters lie within 150 nucleotide of each other within the D-loop (see Figure 1:2). Although consensus elements have been found in lower eukaryote and plant mitochondrial promotors (Jaehning, 1992) mammalian mitochondrial transcrioptional start sites show little sequence conservation. It seems that elements upstream of the initiation sites are important for positioning of the RNA polymerase by binding the transcription stimulatory factor, mtTFI (Parisi & Clayton, 1991).

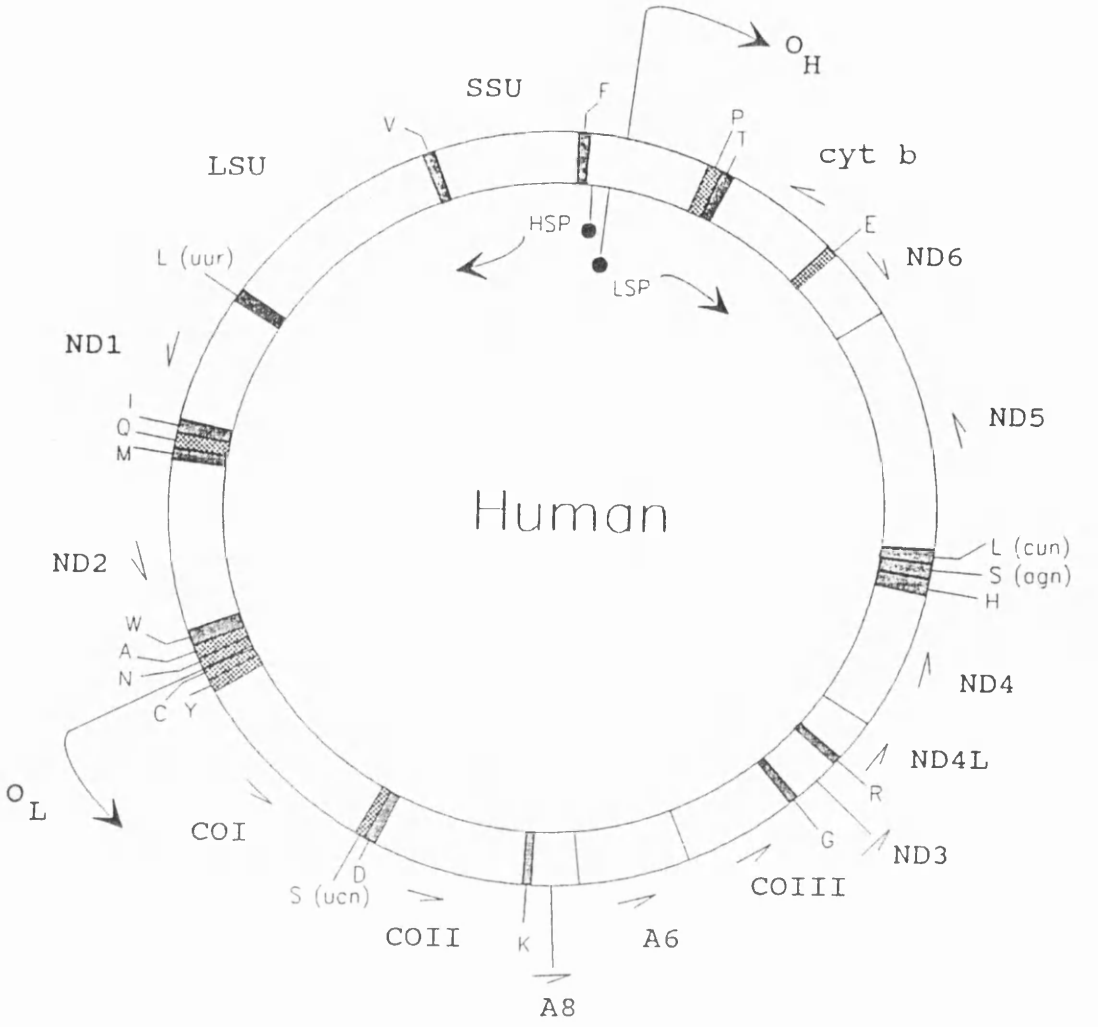


Figure 1:2 Schematic representation of the 16.6 kb human mitochondrial DNA. Coding regions for the 13 polypeptides: ND1, ND2, ND3, ND4, ND4L, ND5, ND6 of Complex 1; cytochrome *b* of Complex III; COI, COII & COIII of Complex IV and ATPase 6 and 8 of Complex V. The 22 tRNAs are represented by their single letter code; the 12S rRNA by SSU and 16S rRNA by LSU. O_H and O_L are the origins of heavy and light strand replication respectively. Arrows indicate direction of transcription or replication.

The H strand and L strand are transcribed as probably genome-length polycistronic transcripts: the H strand encodes the two ribosomal RNAs, 12S and 16S, 14 tRNAs and 12 of the 13 polypeptides; the L strand codes for 8 tRNAs and only 1 polypeptide. Punctuation of the genome with tRNAs is thought to allow processing of the polycistronic transcript at the 5' and 3' ends of the tRNAs (Attardi, 1985). An RNaseP-like enzyme which cuts at the 5' end of tRNAs has been identified in HeLa cells (Doersen et al, 1985); the processing enzyme for the 3' end of tRNAs has not been identified.

Mitochondrial biogenesis requires co-ordination of nuclear and mitochondrial gene expression. The remaining mitochondrial proteins are encoded by the nuclear DNA. The genes for these are transcribed in the nucleus, their mRNAs translated in the cytosol and the polypeptides transported into the mitochondrion (for review see Hartl et al 1989). Then, in concert with the mitochondrially encoded subunits, they are assembled to form the complexes of the oxidative phosphorylation pathway. Control of mitochondrial gene expression is thought to be via nuclear encoded factors: mtTFA, the product of a nuclear gene, stimulates transcription from the two mitochondrial promoters. This gene is itself under the control of so-called nuclear respiratory factors (NRF), transcription factors which thus provide nuclear regulation at mitochondrial transcription and (indirectly) translation (Virbasius & Scarpulla, 1994).

Until recently human mitochondrial DNA was thought to be inherited exclusively from the mother (Giles et al 1980). However, a report by Gyllenstein et al (1990) provides evidence, from studies in mice, that mitochondrial DNA from the father is inherited at a low level.

1:2 MITOCHONDRIAL DNA POLYMORPHISMS

Variations in mitochondrial DNA occur naturally in all human populations (Vilkki et al 1988), but most individuals appear to be homoplasmic, i.e. have only one type of mitochondrial DNA. A mixture of more than one type of mitochondrial DNAs, i.e. wild-type and mutant, is known as heteroplasmy.

The speed of evolutionary divergence of human mitochondrial DNA is rapid in comparison to that of nuclear genes. This property, in addition to the virtually exclusive maternal inheritance of mitochondrial DNA, allows sequence differences, i.e. polymorphisms to be extensively used to measure rates of divergence of the various populations within the human species. Cann et al (1987) calculated that the average number of base pair differences between two

human mitochondrial genomes is 9.5. In contrast Merriweather et al (1991) stated that two individuals would differ on average by 0.4% which gives a figure of 66 base changes.

Using such comparisons Cann et al (1987) traced the human mitochondrial genomic ancestor to Africa approximately 200,000 years ago; Merriweather et al (1991) are in agreement with this. This time scale is contested by paleoanthropologists who estimate the time of human origin, by means of archeological data, dental and bone remains and nuclear genetic factors, as much earlier. Many populations have now been studied using mitochondrial DNA polymorphisms: Asian (Bonne-Tamir et al 1986); American (Torroni et al 1992); Russian (Lemza et al 1992).

The human mitochondrial DNA sequence- the so-called "Cambridge" sequence - was published in 1981 (Anderson et al) and is the sequence to which all other sequence is compared. However, in the last few years a number of differences from the Cambridge sequence have been repeatedly reported. See Table 1:1. Explanations given for these discrepancies are that there are mistakes in the reading of the original sequences, or that the Cambridge sequence was obtained using a source of DNA containing an unusual array of polymorphisms. The sequence published in 1981 was a mosaic of sequence from a number of human placentas and HeLa cells (Wolstenholme, 1992) and this could perhaps account for some of the differences.

When a large sequencing project is undertaken these differences have to be borne in mind and where an apparently novel polymorphism is associated with disease the possibility that it is a naturally occurring, though rare, polymorphism must be considered.

1:3 MITOCHONDRIAL DISEASE

In recent years an increasing number of diseases have been found, characterised by identifiable defects in the oxidative phosphorylation pathway. These diseases are known collectively as the mitochondrial encephalomyopathies and the range of symptoms is very wide: deafness, optic atrophy, muscle weakness, dementia, cardiac defects and so on. Advances in molecular genetics have revealed that many of these diseases are associated with mutations in the mitochondrial genome or in nuclear genes involved in mitochondrial gene expression. Differential tissue expression of these mutations is thought, in part, to result from the variable reliance of tissues on mitochondrial energy production: not all tissues have the same oxidative phosphorylation requirement. The central nervous

Gene	Position	Change on sense-strand	Amino Acid
12S rRNA	1438	A->G	-
ND1	3423	G->T	val-conserved
ND2	4769	A->G	met-conserved
	4985	G->A	gly-conserved
ATPase 6	8860	A->G	thr->ala
ND4	11335	T->C	asn-conserved
	11719	G->A	gly-conserved
ND5	13702	G->C	gly->arg
ND6	14199	G->T	pro->thr
	14272	G->C	phe->leu
	14365	G->C	val-conserved
	14368	G->C	phe->leu
Cyt*b*	15326	A->G	thr->ala

Table 1:1 Discrepancies from the "Cambridge" sequence of human mitochondrial DNA reported in normal individuals. Compiled from Howell et al (1992) and Marzuki et al (1992).

system (CNS) is most dependent on oxidative phosphorylation and this could explain why there are so many neurological symptoms in these diseases. However, as symptoms vary widely and are to some extent correlated with the particular mutation, this is clearly not the whole explanation.

In an attempt to make a more systematic study of mitochondrial diseases Shoffner & Wallace (1990) produced a classification system:

Class I nuclear DNA mutations
Class II mitochondrial DNA point mutations
Class III mitochondrial DNA deletions and duplications
Class IV of unknown inheritance

With a similar aim Zeviani et al (1990) proposed that mitochondrial diseases be classed into three categories depending on aetiology:

1) defective transcription or translation of mitochondrial proteins coded by nuclear genes.
2) mutations of mitochondrial DNA.
3) mutations of nuclear genes which control mitochondrial gene expression.

The latter categorisation is more meaningful but it is probable that many of the mitochondrial diseases involve a nuclear gene defect acting in conjunction with or sometimes causing a mitochondrial DNA mutation. Consequently most diseases do not fit neatly into one category and I shall discuss a number of the best documented diseases with regard to the mitochondrial mutations identified and their putative role in the pathology.

1:4 LEBER'S HEREDITARY OPTIC NEUROPATHY (LHON)

Leber's Hereditary Optic Neuropathy is a form of optic atrophy which was first described in 1871 by a German ophthalmologist - Theodore von Leber - (Leber 1871) as a disease of the central nervous system. It is characterised by acute bilateral blindness, the age of onset of which is around 20 years; there is found to be a sex bias in that only 15% of those affected are female (Went et al 1975). As early as 1935 (Imai et al) studies were being undertaken into the genetic basis of the disease and it was suggested to be a case of "cytoplasmic inheritance". However, in 1970 Douglas Wallace studied a large pedigree of 101 individuals, a number of whom suffered from LHON, and suggested that the maternal pattern of inheritance was consistent with vertical transmission of a virus. Erickson (1972) also observed that the disease exhibited a non-Mendelian pattern of

inheritance and that it was exclusively maternally inherited. In 1984 Nikoskelainen et al suggested that since it was known that mitochondrial DNA was exclusively maternally inherited, it may play a role in the disease.

1:4:1 The "Classical mutation" in LHON

It was not until 1988 that Wallace et al found a mutation in the mitochondrial DNA of patients suffering from LHON, which correlated strictly with the disease. In order to minimise the chances of associating LHON with a rare ethnic polymorphism (most LHON pedigrees are caucasoid) a large pedigree of black LHON patients were examined for candidate mutations. Restriction enzyme analysis of PCR products of mitochondrial DNA from lymphoid and muscle tissue excluded large deletions as the cause of LHON. By cloning and sequencing, 25 base substitutions were found, 8 of which altered amino-acids, and Wallace set out criteria for implication of a mitochondrial replacement mutation in LHON:

If it:

1. changed a highly conserved amino acid
2. was frequently found in LHON
3. could not be found in normal controls

Six of the 8 base substitution mutations were excluded as candidate mutations using these criteria. A change at np 9163 was found to convert a highly conserved Valine to an Isoleucine in ATPase 6. However, in hybridisation studies this mutation was not found in all LHON patients but only in one pedigree, leading Wallace to conclude that this mutation did not correlate with LHON.

Finally the G->A transition at np 11778, which causes the replacement of an Arginine residue by a Histidine residue at a highly conserved site in the respiratory chain subunit ND4 (Figure 1:3) was found to be the only change which fulfilled his criteria. This base change in the mitochondrial genome destroys an SfaN1 restriction enzyme site and therefore provides a relatively simple screening method. This point mutation was found in 9/11 LHON families and was not present in 45 controls.

Wallace found that individuals carrying this mutation were homoplasmic; Vilkki et al (1989) confirmed this finding in an initial study of 19 Finnish LHON families where they found that 10/19 families had the mutation. Holt et al (1989) reported both wild-type and mutated mitochondrial DNA (heteroplasmy) in the peripheral blood of 4/8 LHON families and correlated the proportions of the

Human (HeLa)	E	R	T	H	S	R	I	M	I	L	S	G
Human (LHON, 11778)	-	-	-	-	-	H	-	-	-	-	-	-
Mouse	-	-	I	-	-	-	T	-	-	M	A	R
Rat	-	-	-	-	-	-	-	-	-	M	A	R
Bovine	-	-	-	-	-	-	T	-	-	-	A	R
Xenopus	-	-	-	-	-	-	A	L	L	-	-	R
Drosophila	-	-	L	G	-	-	S	-	L	I	N	K
Sea Urchin	-	-	S	G	T	-	T	L	A	I	T	R

amino acid 340

Figure 1:3 Partial amino acid sequence of subunit ND4 in human, mouse, rat, bovine, *Xenopus*, *Drosophila* and sea urchin showing conserved Arginine residue at amino acid 340 in different phyla.

mitochondrial DNA types with the severity of the disease. They found that in the families without the 11778 mutation, recovery of vision to a functionally significant extent approximately 4 years after onset of symptoms, was often the case. In addition those patients with the 11778 mutation at high percentage, i.e. > 98% mutant, were worst affected. The symptomless carrier females in the families with the 11778 mutation had levels of heteroplasmy of < 80% mutant.

1:4:2 Mutation at np 3460 in LHON

Huoponen et al (1990) reported that they could not find the previously described ND4 mutation in 9/20 families with members diagnosed as having LHON. This group (Huoponen et al 1991) have subsequently found another mitochondrial mutation in 3 Finnish LHON families. A G->A transition at nucleotide 3460 was observed, in blood mitochondrial DNA, this transition causes substitution of an Alanine by a Threonine in the ND1 subunit of complex I. The homoplasmic mutation abolishes an Aha II restriction site and consequently can also be easily detected. The ND4 mutation np 11778 was not observed in any of these families.

1:4:3 Mutations at np 4160 and np 4136

A number of LHON families do not have any detectable mutation, though the mitochondrial genomes of some of these have not been fully sequenced, obviously there may be mutations present in the parts of the genome not already sequenced: Howell and McCullough (1990) looked at a large Australian family and could find no candidate mutations in ND5, ND4L, ND4 or the contiguous tRNA genes. They suggested that LHON might be genetically heterogeneous, i.e. that a set of related diseases might be being identified and classified as LHON. However, this group (Howell et al 1991) subsequently reported two mutations in the above family, in the mitochondrial gene for the ND1 subunit.

The first mutation found was a T->C transition at np 4160; all members of the family studied appeared to be homoplasmic for the mutation, but only 5 shared symptoms of the disease in a similar way that not all individuals with the 11778 and 3460 mutations show symptoms of disease. This change in the DNA causes a leucine to be replaced by a proline in a small helix of hydrophilic loop of the ND1 protein subunit. Another mutation was identified at position 4136, a homoplasmic A->G transition which results in substitution of cysteine for tyrosine at amino-acid 277 of the ND1 protein subunit. This second mutation was not identified in all family members carrying the first mutation. Of those people carrying both mutations only two were clinically abnormal, but these two exhibited less severe symptoms than other family members with only the np 4160

mutation. Howell et al (1991) suggested that these data support the idea that the second (np 4136) mutation acts as a partial suppressor of the first.

The Queensland family in which this mutation has been identified is one of only a few LHON families in which a biochemical defect has been reported. Parker et al (1989) showed a decrease in Complex I activity in platelet mitochondria in four members of this family.

Approximately fifty percent of LHON pedigrees have been shown to have the np 11778, ND4 mutation, while 15-25% of the remainder carry the ND1, np 3460 mutation.

1:4:4 Additional mutations associated with LHON

Since 1991 a number of other mutations of mitochondrial DNA have been linked with LHON. Table 1:2 shows all the mutations thus far associated with LHON. The changes reported by Johns and Berman in 1991 were also found in controls, albeit at lower frequencies. In addition, the change at np 4216 in ND1 was never found alone, but always in conjunction with either the change at np 13708 or that at np 4917. The G->A change at np 13708 was later reported by Brown et al (1992), who found it in 11778 positive and negative LHON patients; it was also reported at lower frequency in controls. Two additional mutations were reported by Brown et al (1992): one at np 15257 and one at np 15812, both in Cytochrome *b*. They found that all patients with the np 15257 change also had the change at np 13708 and all those with the np 15812 mutation had those np 15257 and np 13708. This led Brown et al to propose that a number of mutations of mitochondrial DNA may work in concert to produce the disease phenotype: the "disease haplotype" theory.

In 1992 Johns et al reported yet another mitochondrial DNA mutation, associated with LHON, which has not been identified in controls. This is a T->C change at np 14484 which changes a methionine to a valine in ND6; it was found in 14 LHON families. It occurred in association with the 13708 change in 10/14 patients and with another mis-sense change at np 3394 in 5/14 patients. This group also proposed the interaction of various independent mutations working together to give rise to disease. Another recently reported mutation associated with LHON is a G->A transition at np 7444 (Brown et al 1992). The mutation was found in 2/22 (9%) of LHON patients who lacked the 11778 mutation and 6/545 (1%) of controls. The change causes the loss of an *Xba*I restriction site and therefore the presence of the mutation is easy to screen.

SUBUNIT	POSITION	NUCLEOTIDE CHANGE	AMINO ACID CHANGE	RESTRICTION SITE CHANGE	REPORTED BY
ND4	11778	G->A	arg->his	*SfaNI* loss	Wallace et al, 1991
ND1	3460	G->A	ala->thr	*AhaII* loss	Houponen et al, 1991
ND1	4160	T->C	leu->pro	-	Howell et all, 1991
ND1	4136	A->G	tyr->cys	-	Howell et al, 1991
ND5	13708	G->A	ala->thr	*Bst*NI loss	Brown et al, 1992
Cyt*b*	15257	G->A	asp->asn	*Acc*I loss	Brown et al, 1992
Cyt*b*	15812	G->A	val->met	*Rsa*I loss	Brown et al, 1992
ND2	5244	G->A	gly->ser	*HpaI*I loss	Brown et al, 1992
ND6	14484	T->C	met->val	-	Johns et al, 1992
ND1	3394	T->C	tyr->his	-	Johns et al, 1992
ND1	4216	T->C	tyr->his	*NlaIII* creation	Johns et al, 1991
ND2	4917	A->G	asp->asn	*Mae*I created	Johns et al, 1991
COI	7444	G->A	term->lys	*Xba* loss	Brown et al, 1992

Table 1:2 Differences from the "Cambridge" sequence (Anderson et al, 1981) associated with Leber's Hereditary Optic Neuropathy

This change alters the termination codon-AGA of cytochrome *c* oxidase subunit I (COI) to become AAA and it is proposed that the COI peptide is extended by 3 amino acids. A decrease in cytochrome-*c* oxidase activity was also reported in EBV-transformed lymphocytes from one of the patients with the np 7444 mutation and on this basis it was proposed that the mutation leads to a partial respiratory deficiency which could lead to the pathogenesis of LHON.

There are a number of patients diagnosed as suffering from LHON who have none of the above mutations; this, and the association with such a large number of different mitochondrial mutations, make the molecular mechanisms leading to the disease difficult to determine. The biochemical basis of the disorder has not been satisfactorily defined: biochemical defects evident in blood may not have relevance to their effects in the optic nerve. An additional factor has frequently been hypothesised in the development of LHON in order to explain why some members of LHON pedigrees develop symptoms, while others remain healthy. Sex linkage, an autosomal nuclear gene, or environmental factors have all been cited and will be discussed below.

1:4:5 Possible X-Linkage in LHON

Although LHON is exclusively maternally inherited, the imbalance of male to female patients (70-80% male, 20-30% female) seems to indicate that some X-linked gene could also be involved in expression of the disease phenotype. It has been proposed that the disease is the result of the combination of a mitochondrial DNA mutation and an X-linked gene. Chen et al (1989), in a multipoint linkage analysis, found no linkage between LHON and 15 X-chromosome markers in 3 LHON families. In a 2 point linkage analysis Vilkki et al (1991) found tentative evidence of linkage to locus DXS7 on the proximal arm of the X-chromosome to which other hereditary eye diseases have been mapped: retinitis pigmentosa and congenital stationary night blindness (Gal et al 1989).

Bu and Rotter in 1991 proposed, based on segregation analysis, that LHON is a two locus disorder; one mitochondrial, one X-chromosome linked. They proposed that females affected by LHON are so due to X-inactivation and that these females would be heterozygous for the defective nuclear gene.

However, in 1993 the Finnish group re-evaluated their linkage data (Juvonen et al 1993), re-examining the families previously studied and including a number of additional pedigrees. The result of this exhaustive study was that they could not confirm the previously reported linkage and they concluded that the reasons for the male bias in LHON remain unknown.

1:4:6 Environmental Factors and LHON

Other factors which could influence the sexual bias in presentation of the disease should also be taken into account. Hormonal involvement is the obvious one, as the age of onset is usually in late adolescence when great hormonal changes are taking place. It is not known if there is some hormonal influence on the manifestation of the disease phenotype which is greater than males than in females.

In the past the suggestion was made that a defect in cyanide metabolism could be involved in the aetiology of LHON (Wilson, 1965). The enzyme Thiosulphate-sulphur transferase (also called Rhodanese) is a mitochondrial matrix enzyme which detoxifies cyanide. Since smoking increases the level of cyanide in the blood stream some studies were made on the effect of smoking on the onset and progression of the disease. However, no convincing correlation was ever found and the idea has largely been abandoned (Nikoskelainen 1984). What the pathogenic mechanisms are in the development of LHON, what role the mutations described here play in the damage to the optic nerve remains unknown.

1:5 MITOCHONDRIAL ENCEPHALOMYOPATHY WITH LACTIC ACIDOSIS AND STROKE LIKE EPISODES (MELAS)

Mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS) is a second, well-documented mitochondrial disease. The principal symptoms are stroke-like episodes, muscle weakness and retinal degeneration (Kobayashi et al 1987); elevated levels of lactate and reduced NADH-CoQ reductase (Complex I) activity were found in the CSF of two patients (Kobayashi et al, 1987). These two biochemical defects were thought to play a role in the disease. Using the polymerase chain reaction (PCR) and sequencing strategies, an A->G transition at np 3243 of the mitochondrial DNA, in the gene for $tRNA_{Leu(UUR)}$ was found in MELAS patients (Goto et al 1990; Kobayashi 1990). This mutation causes the gain of an *ApaI* restriction enzyme site and is therefore simple to detect by restriction fragment length polymorphism (RFLP) analysis.

In the case of the np 3243 mutation heteroplasmy is always the case, with populations of mutant mitochondrial DNA in muscle, from 50% to 92%. Figure 1:4 illustrates the proposed secondary structure of the mitochondrial $tRNA_{Leu(UUR)}$ and shows the position of the 3243 mutation: this site is conserved in mitochondrial tRNAs from human to sea urchin (Goto et al, 1990).

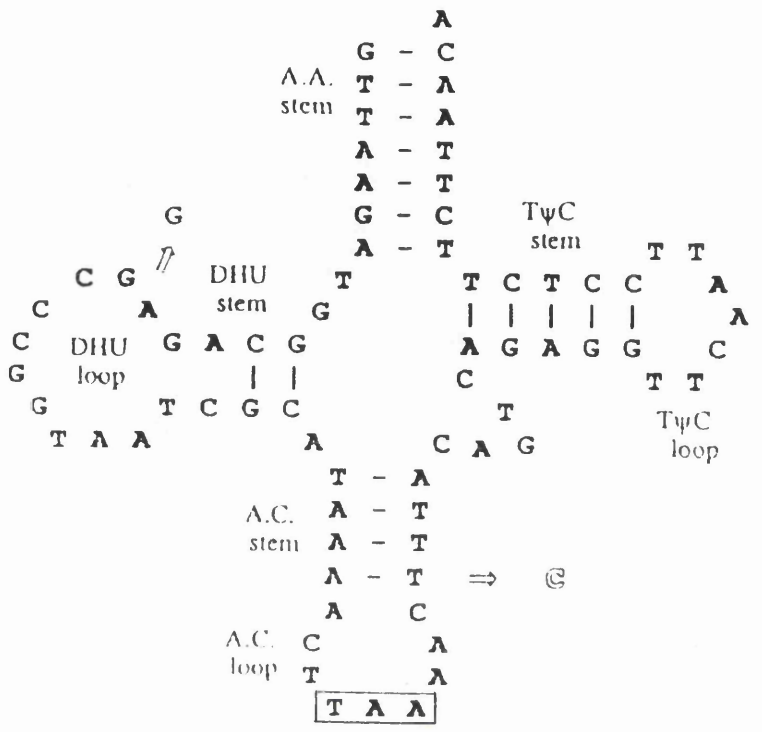


Figure 1:4 Secondary structure of mitochondrial $tRNA_{Leu(UUR)}$ showing the disease associated mutations at np 3243 and 3271.

Taken from Goto et al (1991).

Hess et al (1991) observed that the mutation lies in the middle of sequences involved in transcription termination of the mitochondrial 16S rRNA. Although the human mitochondrial genome is transcribed as a polycistronic molecule there are relatively more rRNA in comparison to other RNA molecules. The boundary of the genes for 16S rRNA and $tRNA_{Leu(UUR)}$ is the major determinant in the control of the relative synthesis. A partially purified protein of 34 kD was found to bind to a tridecamer sequence at the 16S rRNA/$tRNA_{Leu(UUR)}$ boundary. When the mutation at np 3243 was present in the DNA substrate the binding of the protein was decreased by approximately 50%. In addition an increase in the amount of full length run off transcript, with little or no prematurely terminated transcript, was observed when the $tRNA_{Leu(UUR)}$ mutation was present. The conclusion was that the mutation prevents binding of the termination factor and therefore termination at the 16S rRNA/$tRNA_{Leu(UUR)}$ boundary. Hess et al (1991) have suggested that the molecular defect leading to the symptoms associated with MELAS may be the lack of production of the correct type and/or quantity of ribosomal RNA, in relation to the levels of other mitochondrial transcription products, in the affected tissues. In contrast to this, Moraes et al (1992) measured the steady state ratios of mRNA:rRNA *in vitro*, by *in situ* hybridisation of muscle biopsy sections. They found that some MELAS patients had increased mRNA:rRNA but others did not. In addition similar increases in mRNA:rRNA were also found in patients suffering from MERRF, who do not have the np 3243 mutation but do have a mutation at np 8344 (see below). Moraes et al concluded that the np 3243 mutation in MELAS causes no specific changes in steady-state ratios of mRNA:rRNA.

Only 75% of MELAS patients have the np 3243 mutation, and other mitochondrial DNA mutations have been associated with some cases of MELAS. Goto et al (1991) reported a T->C transition at np 3271 which is also in the $tRNA_{Leu(UUR)}$ gene. This polymorphism was not found in any of the 46 patients with other mitochondrial diseases nor in 50 controls. The mutation lies outside the area involved in transcription termination, giving support to the idea that tRNA abnormalities, which may cause altered binding to the ribosome, or its cognate aminoacyl-tRNA synthetase, or non-charging or mischarging of the $tRNA_{Leu(UUR)}$ molecules, are involved in the mechanisms leading to the disease state. This mutation has not been reported by others.

The mutation at np 3243 has also been found in a number of families in which diabetes mellitus (DM) and/or sensorineural deafness is maternally inherited (Reardon et al, 1992; van den Ouweland et al, 1992). The differences in disease phenotype in patients with this mutation in $tRNA_{Leu(UUR)}$ probably reflects

differential tissue expression of the mutation under the control of mitochondrial or nuclear factors.

1:6 MYOCLONIC EPILEPSY WITH RAGGED RED FIBRES (MERRF)

Myoclonic Epilepsy with Ragged Red Fibres (MERRF) is also known as Fukuhara disease. The symptoms are heterogeneous but the main clinical features are ataxia, myopathy with ragged-red fibres and myoclonic epilepsy. Other symptoms reported are deafness, dementia and mild cardiomyopathy. Wallace et al (1988) noted that the syndrome was maternally inherited implying that there could be a defect in the mitochondrial DNA. When sections of muscle biopsies are stained with modified Gomori trichome, structures referred to as Ragged Red Fibres are observed (Moraes et al 1989). Electron microscope studies show these to be aggregates of abnormal mitochondria with paracrystalline arrays and degeneration of the cristae (Wallace et al 1986). These structures are, in fact, a common symptom of many of the mitochondrial encephalomyopathies.

Examination of mitochondrial DNA from the muscle of MERRF patients using PCR (Saiki et al 1988) and direct sequencing revealed a point mutation at np 8344 (Shoffner et al 1990). This mutation was found to be an A->G transition which alters an evolutionarily conserved base in the TψC loop of the $tRNAs_{Lys}$ gene, and creates a *Cvi*JI restriction enzyme site. The creation of this site allows the mutation to be easily detected and both wild-type and mutated mitochondrial DNA (heteroplasmy) in varying proportions has been found in MERRF patients.

Silvestri et al (1993) reported levels of heteroplasmy between 56% and 100% in eleven MERRF patients; in this study the mutation was identified in both blood and muscle. No correlation was found between the severity of disease and the percentage of mutated mitochondrial DNA molecules. Wallace et al in 1988 had reported defects in Complexes I and IV in MERRF patients, but the more recent report by Silvestri identified a significant decrease only in Complex IV. However, no correlation was found between the biochemical defect and the level of heteroplasmy.

1:7 NEUROGENIC MUSCLE WEAKNESS, ATAXIA AND RETINITIS PIGMENTOSA (NARP)

Neurogenic muscle weakness, ataxia and retinitis pigmentosa (NARP) has been identified as a variable combination of developmental delay, retinitis pigmentosa, dementia, ataxia, muscle weakness and sensorineuropathy. In 1990 Holt et al identified a maternal pedigree in which 4 members were seen to have two

populations of mitochondrial DNA in blood and muscle. One population had a previously unreported T->G point mutation at np 8993, changing a highly conserved leucine to an arginine in the ATPase 6 gene. Some correlation between the degree of heteroplasmy and clinical severity was observed.

Tatuch et al (1992) identified this heteroplasmic np 8993 change in skin, brain, kidney and liver of an infant whose symptoms were consistent with Leigh's disease (a fatal neurodegenerative disease similar to NARP, but identified in infants). This mutation has now been found in a number of families and its identification has been used in prenatal diagnosis of the syndrome (Harding et al 1992).

Differential tissue expression and threshold levels of mutant mitochondrial DNA may play some role in the development of the disease. Tatuch et al (1992) identified unaffected members of a family with NARP, who had up to 71% mutant mitochondrial DNA in fibroblasts but only 39% in lymphoblasts. It has been proposed that the insertion of an arginine residue in a hydrophobic sequence of the ATPase 6 subunit of ATP synthetase interferes with the H+ channel formed by ATPase subunits 6 and 9, leading to diminished ATP synthesis.

1:8 DISEASES ASSOCIATED WITH REARRANGEMENTS OF MITOCHONDRIAL DNA

Rearrangements of the mitochondrial genome in the form of partial duplications and deletions have been reported in a wide range of mitochondrial encephalomyopathies. Holt et al (1988) used multiple restriction enzyme analysis of mitochondrial DNA extracted from muscle to identify the presence of deletions. They reported 9/25 patients with mitochondrial myopathy had detectable levels of mitochondrial DNA molecules deleted by up to 7 kb; they later reported (Holt et al 1989) that 40% of patients with mitochondrial myopathies have these large deletions. Poulton et al (1989), using similar restriction enzyme analysis, observed large scale duplications (around 8 kb) in 2 patients with mitochondrial myopathy. It has since been realised that it can be difficult to differentiate between partial duplications and partial deletions. It is only possible to identify a duplication when a restriction enzyme which cuts uniquely at a site within the deleted region is used (Poulton et al, 1994).

1:8:1 Kearns Sayre Syndrome/Chronic External Ophthalmoplegia

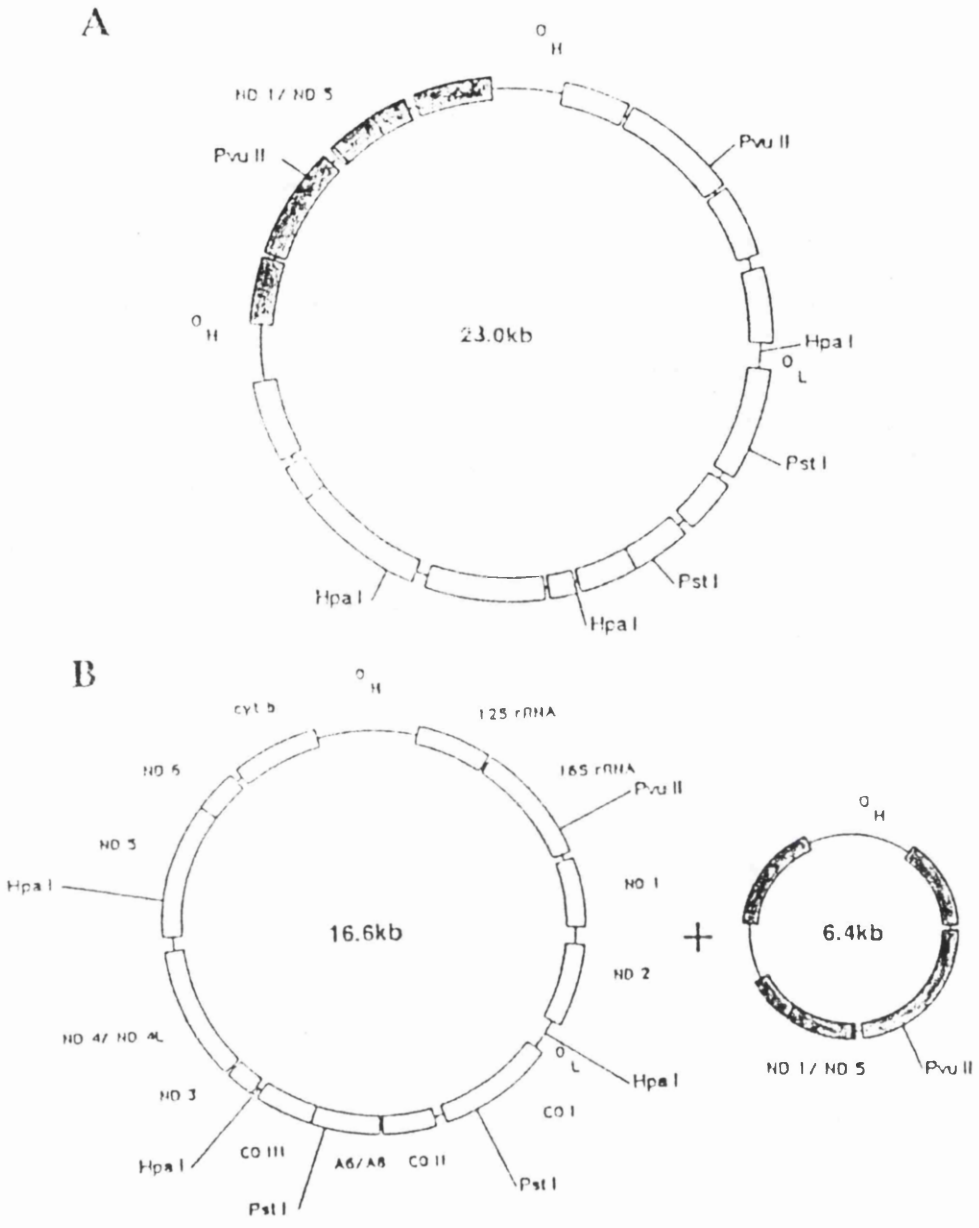
The disease on which most work on the study of rearrangements of the mitochondrial DNA has been done is Kearns/Sayre Syndrome (KSS). This is a

disease chracterised by progressive external ophthalmoplegia (paralysis of the external eye muscles), cardiac defect, sensorineural deafness and pigmentary retinopathy with the mean age of onset around 20 years. Chronic progressive external ophthalmoplegia (CPEO) is a milder form of KSS which presents later in life. Microscopically, ragged red fibres are seen in muscle tissue (Shoffner et al 1989) these contain large amounts of deleted mitochondrial DNA (Shoubridge et al, 1990); biochemically there is a reduction in respiratory chain enzyme activity, in particular of cytochrome *c* oxidase. Various large scale deletions of the mitochondrial DNA have been reported in different patients:- around 5 kb - Lestienne et al 1988; 1.3->7.6 kb (but an identical 4.9 kb deletion in 11 patients) - Moraes et al 1989; 4.9 kb - Shoffner et al 1989; a number of different deletions in different patients but a common 5 kb in > 33% patients - Schon et al 1989.

Heteroplasmy is always the case as may be easily predicted since if all the mitochondrial genomes in a patient were deleted to this extent the oxidative phosphorylation pathway would be non functioning and death would result. The deletions are most commonly from the region spanning np 8500 to around np 13000 which removes all or part of the genes for ATPase 6/8, COIII, ND3, ND4L/4, ND5, ND6 and a number of tRNA genes.

These reported deletions had been thought to be sporadic but Poulton et al in 1991 reported a patient with KSS and two asymptomatic relatives with the same mitochondrial DNA deletion. Using PCR they observed the so called common deletion of 5 kb in the blood and muscle of both the patient and relatives. Zeviani et al (1990) also reported a large pedigree in which the inheritance of multiple deletions in the mitochondrial genome appears to be autosomal dominant. Cormier et al (1991) noted that carriers of such multiple deletions were not always clinically affected and, in agreement with Zeviani et al (1990), suggested that a mutation in a nuclear gene triggers multiple deletions of the mitochondrial genome.

The realisation by Poulton et al (1994) that there can be a failure to discriminate simple deletions from multiple rearrangements in mitochondrial DNA prompted a reinvestigation of a number of KSS and CPEO patients. Figure 1:5 shows a schematic representation of a partial duplication and partial deletion of human mitochondrial DNA molecules. The 6.4 kb partial deletion could result from recombination between the duplicated portion of the 23 kb partial duplication molecule. Using appropriate restriction enzymes Poulton et al (1994) found that 10/10 KSS patients had both deletions and duplications while 8/8 CPEO patients had only duplications. The role of the different rearrangements in the development of different phenotypes is unknown.



Altered from Dunbar et al. 1993.

Figure 1:5 Schematic diagram showing A. a partially duplicated mt DNA molecule. B. normal mitochondrial genome and the molecule produced by a partial deletion of the molecule shown in A.

The mechanisms by which rearranged molecules arise are also unknown: one theory is of intramolecular recombination (Mita et al, 1990), although this would be difficult to reconcile with the presumed lack of a DNA repair system in human mitochondrial DNA. Another theory is one of replication slippage (Shoffner et al, 1989) due to direct repeats at the breakpoint of the rearrangement. A nuclear gene defect could be implicated in either theory to explain the deletions which are apparently inherited. The sporadic deletions may result from a mistake in mitochondrial DNA replication during some crucial developmental stage, in a particular cell-type.

1:8:2 Diabetes and deafness

Ballinger et al (1992) studied a pedigree in which diabetes mellitus (DM) was inherited, along with deafness, via the maternal line. This combination of symptoms was inherited over 3 generations. The mitochondrial DNA of muscle from the index case was found to be heteroplasmic: 31% normal, 69% deleted by 10.4 kb, i.e. only 6.1 kb in length. By direct sequencing the deletion was found to be between np 4308 and np 14874. The muscle of the index case also showed severe reduction of all the complexes of oxidative phosphorylation. Surprisingly there were no reported muscle symptoms in this family. The effect of this deletion on mitochondrial protein synthesis was measured using EBV-transformed lymphoblast cell-lines and primary myoblast cell-lines. The lymphoblast cell-line, with 53% of the mitochondrial DNA molecules deleted, showed a decrease of 52% in ^{35}S-Methionine incorporation into mitochondrial translation products. The myoblast cell-line from the same patient had only 17% deleted mitochondrial DNA molecules and mitochondrial protein synthesis at a normal level. Ballinger et al (1992) concluded that the deletion was associated with decreased mitochondrial protein synthesis. The mechanism by which this results in the symptoms observed is unknown.

This three generation family was subsequently re-examined (Ballinger et al, 1994) and found to have at least two species of rearranged mitochondrial DNA molecules: a duplication and deletion dimer. The duplicated molecule has a 6.1 kb insertion duplicating the origin of heavy strand replication but not the origin of light strand replication. The deleted molecule comprises tandem copies of the 6.1 kb segment giving a deletion dimer. The proportions of duplicated and deleted mitochondrial DNA vary greatly within the family, but it seems that transmission is of a mitochondrial DNA duplication. The deleted molecules accumulate in somatic tissues, and may cause a decline in oxidative phosphorylation. When ATP production falls below a critical level in pancreatic β cells diabetes mellitus is presumed to result.

Dunbar et al (1993) identified a partially duplicated mitochondrial DNA in the blood and muscle of a patient and his mother, both of whom had late onset DM. The duplicated molecule was found (by various restriction enzyme digestions) to extend from np 13445 (ND5) to np 3318 (ND1), flanked by an imperfect direct repeat.

The development of diabetes in families in which there are partial duplications in mitochondrial DNA, in addition to those families with the np 3243 mutation in $tRNA_{Leu(UUR)}$ (Reardon et al, 1992; van den Ouweland et al, 1992) implies a possible common pathogenic mechanism.

1:9 DISEASES ASSOCIATED WITH MITOCHONDRIAL DNA DEPLETION

Recently a small number of patients have been identified who have a quantitative change in mitochondrial DNA, in contrast to the qualitative changes, i.e. point mutations, rearrangements, so far described. Moraes et al (1991) described four infants who were found to have a severe depletion of mitochondrial DNA in tissues in which symptoms of mitochondrial disease were observed. The levels of mitochondrial DNA depletions in these patients correlated well with decreased Cytochrome *c* oxidase (COX) activity: one patient had COX activity which was only 2% of control in muscle, and 7% in liver, which correlates with 2% and 12% of control mitochondrial DNA levels.

All 4 patients in this study died in early infancy presumably due to an inability of tissue to survive with virtually no oxidative phosphorylation. Mazziotta et al (1992) also described a child with a fatal, in this case liver, disease in which there was severe depletion of mitochondrial DNA. Biochemical studies indicated marked deficiencies of respiratory chain complexes I, III and IV activity in the liver.

It has been proposed that mitochondrial DNA depletion is caused by a nuclear gene defect impairing mitochondrial DNA replication. In order to investigate this hypothesis Bodnar et al (1993) studied fibroblasts from a patient who had a similar fatal mitochondrial disease, in which mitochondrial DNA was greatly depleted. These cells in culture had a requirement for uridine and pyruvate, which is a characteristic of rho° cells.

Tissue culture cells will become rho°, i.e. devoid of mitochondrial DNA, after long term exposure to low concentrations of ethidium bromide (King and Attardi 1989). Bodner et al (1993) fused enuncleated fibroblasts (cytoplasts) from a patient with normal fibroblasts and found that normal levels of patient mitochondrial DNA were restored: the implication being that a normal nuclear gene product can restore replication of the patient's mitochondrial DNA.

Nuclear DNA involvement would be consistent with the proposed mechanisms of inheritance of mitochondrial DNA depletion, which is either autosomal recessive (Moraes et al 1991) or autosomal dominant with incomplete penetrance (Mazziotta et al 1992). Mitochondrial DNA depletion is a recent and rarely reported phenomenon. It is probable in future, that levels of mitochondrial DNA will be measured in patients who present with clinical symptoms of combined respiratory chain complex deficiencies. This, in addition to examination for the presence of mitochondrial DNA mutations will further elucidate the disease mechanisms in this heterogeneous group of patients.

1:10 METHODS OF INVESTIGATION OF MITOCHONDRIAL MUTATIONS IN RELATION TO DISEASE

Mutations, of the mitochondrial genome, associated with specific diseases have been investigated in a number of ways. In many studies mitochondrial DNA has been prepared from muscle biopsies (Moraes et al, 1992) or blood (Hammans et al, 1991) and sequence changes investigated by RFLP analysis.

Many studies have been carried out using cell-lines created from tissue, of various types, obtained from patients. Fibroblasts (Robinson et al, 1987), myoblasts (Kobayashi et al, 1992) have all been used. The advantages are that *in vitro* studies of oxidative phosphorylation capacity, mitochondrial DNA, RNA and proteins can be easily carried out. The disadvantage of studies using cell-lines is in the tissue-specificity of many mitochondrial diseases. For example, the decrease in cytochrome-*c* oxidase activity found in lymphoblastoid cell-line prepared from a LHON patient (Brown et al, 1992) is difficult to relate to the clinical phenotype of LHON.

Another method has been the use of cybrid cell-lines which are produced by fusion of two different kinds of cell, one of which has been enucleated. This method was particularly useful in early studies of chloramphenicol resistance (Bunn et al, 1974). More recently cybrids have been made between normal cells which are rho° and enucleated cells with mutant mitochondrial DNA. These have been used in the study of MELAS (Chomyn et al, 1992; King et al, 1992)

and have provided a means to study the genetic and biochemical effects of the np 3243 mutation. Obviously combinations of the above methods will provide most information regarding the relationship of specific mitochondrial DNA mutations to particular diseases.

1:11 INHERITED DEAFNESS

There are many known or suspected environmental causes of deafness: noise, infection, antibiotics, vaccination. Congenital deafness accounts for approximately 50% of childhood hearing loss and this can be divided into two categories: syndromic and non-syndromic. There are a large number of syndromes in which deafness is a major symptom, e.g. Usher Syndrome and Waardenburg Syndrome. Most congenital deafness is non-syndromic, and it can be tentatively categorised into autosomal recessive, which accounts for 60-70% of cases, autosomal dominant, 20-30% of cases and X-linked, 2% of cases (Jaber et al 1992). A recent addition to this category is maternally inherited deafness. As will be described later in this thesis, it is probable that some inherited deafness previously categorised as above will in fact turn out to be maternally inherited.

In general, all pedigree analyses involving deafness are fraught with problems as deaf individuals tend to associate with and partner other deaf people. Determining the mode of inheritance is also impeded by the fact that most pedigrees are too small to determine unambiguously the mode of inheritance.

The identification of genes causing deafness in the mouse is likely to be fruitful in identifying human homologues. Large families of mice can be generated in a relatively short time providing a powerful resource for identifying candidate genes by positional cloning (K. Steel, 1993). In addition, the structure and function of the mouse and human inner ear are very similar. The inner ear pathology observed in genetic deafness is very similar as is involvement of other features such as deafness with pigmentation defects or with digit malformations. Waardenburg syndrome is one disease in which studies in mice have led to identification of the human homologue.

Mutations in the Pax-3 gene in mouse result in a mouse phenotype called Splotch which has coat pigmentation alterations and is deaf. The homologue in humans, PAX-3, and mutations in exons 2 and 4 of this gene have been identified in Waardenburg syndrome (A. Read, 1993). The use of mice in the study of the genes associated with deafness will obviously result in the identification of genes responsible. A major problem could be in the study of less severe forms of deafness, as is the case in the family described in Chapter 4 of this thesis. It is

unlikely that mild to moderate hearing loss, which can be debilitating in humans, could be easily identified in mice.

1:11:1 Sensorineural hearing loss in Mitochondrial Disease

The symptom of deafness is one often described in mitochondrial disease and a number of families have now been identified in which deafness inherited via the maternal line is the sole or predominant feature (Bu et al 1993; Elverland and Torbergsen 1991). In addition, maternally inherited deafness has been found in combination with other symptoms - most commonly diabetes (see Section 1:7); but also myoclonus (Vaamonde et al 1992) and epilepsy (Kobayashi et al 1987). Various types of mitochondrial mutation have been identified in these patients: large deletions or duplications, point mutations of tRNA genes or of the 12S rRNA genes. The pathological mechanisms by which this wide range of molecular defects in mitochondrial DNA cause the development of sensorineural hearing loss have not been elucidated.

1:11:2 Antibiotic-induced deafness

Streptomycin is known to cause deafness when administered in high doses and over long periods of time, as is the case in the treatment of pulmonary tuberculosis. In fact all of the aminoglycoside antibiotics are known to be ototoxic; the mode of action is thought to involve damage to the Organ of Corti (part of the cochlea) hair cells (Ballantyne 1970), in addition to toxicity to the 8th cranial nerve. Characteristic initial effects of such antibiotic treatment is loss of hearing at high frequencies. Since 1964 when the first family study was reported (Prazic et al 1964) there have been a number of reports of families in which susceptibility to streptomycin is inherited. Johnsonbaugh et al (1974) and Viljoen et al (1983) both studied families in which streptomycin had been administered and both concluded that the inheritance was autosomal dominant.

There have now been a number of studies in which the predisposition to become deaf after treatment with aminoglycosides has been found to be maternally inherited. Higashi in 1989 identified 28 families where this was the case; in 1991 Hu et al analysed 36 Chinese pedigrees and found similar results. Prezant et al (1993) have discovered a point mutation at np 1555 in the 12S rRNA in this latter group of families. This A->G change at np 1555, which has not been identified in normal controls, is in a highly conserved region of the 12S rRNA which is very similar to a region of *E. coli* 16S rRNA at which aminoglycosides are known to bind. They postulate that the mutation lengthens the binding site for the

aminoglycoside causing increased binding and a consequent increase in susceptibility to such antibiotics.

1:12 AIMS OF PROJECT

The aim of this study was to identify any mutations of human mitochondrial DNA which are to be found only in association with sensorineural hearing loss. The project was initiated after a patient previously diagnosed as having LHON developed sensorineural hearing loss. This was a previously unreported combination of symptoms.

At the beginning of the project it had not been established whether this patient had any of the previously reported mitochondrial mutations associated with LHON. Sequencing of the entire mitochondrial genome was undertaken to establish which, if any, LHON mutation was present in addition to other mutations which could play a role in the development of deafness.

Patients suffering from sensorineural hearing loss of no known aetiology were selected to be screened, by RFLP analysis, for the presence of known mutations. This in combination with the complete sequence of the LHON patient would indicate whether any of these mutations should be further investigated to establish their role in the aetiology of deafness.

Patients suffering from AAID were included in a limited study of firstly, a mutation already identified in maternally inherited sensorineural hearing loss at np 1555 and secondly, a mutation in the *Xba*I site at np 7440 already identified in LHON.

One of the sensorineural hearing loss patients was studied in much more detail as a result of her apparently maternally inherited deafness, in addition to the discovery of a previously unreported mutation in her mitochondrial DNA. Sequencing of the entire mitochondrial genome of this patient was undertaken in a similar way to that of the LHON patient in order to establish whether the two had any mutations in common and as such could be implicated in the development of deafness.

The role of any mutations found would be investigated by *in vitro* studies using lymphoblastoid cell-lines created using patient lymphocytes. This strategy was designed to identify any mitochondrial mutation which may be responsible, in combination with nuclear genetic or environmental factors, for the development

of hearing loss. It would also yield preliminary data regarding the molecular mechanisms involved.

CHAPTER 2 MATERIALS AND METHODS

2:1 SOURCES OF CHEMICALS AND ENZYMES

Chemical/enzyme	Source
General chemicals and solvents	BDH, ICN
Culture media	GIBCO BRL, ICN
Biochemicals	Sigma, BRL
Agarose	BRL
Film	Dupont
Radiochemicals	NEN (Dupont)
Restriction enzymes	BRL, New England Biolabs, Boehringer Mannheim, Promega
Modifying enzymes	Boehringer, BRL, Promega
PCR product purification systems	Promega, Qiagen
Magnetic beads	Dynal
Biotin Phosphoramidite	Cruachem
Digoxigenin-labelling and detection	Boehringer Mannheim
Sequenase Version 2 sequencing kit	United States Biochemical
Silver staining kit	BioRad
Long Ranger & MDE gel	Hoefer Scientific
Sequencing gel solution	NBL
NA45 paper	Schleicher & Schull

B&W Buffer (2X)	10 mM Tris/HCl pH 7.5, 1 mM EDTA, 2 M NaCl
COX Assay Buffers	40 mM KPi Buffer (see below)with 0.2% Saponin, 16 mg Cytochrome *c* in 20 ml of 40 mM KPi Buffer, 6.5 mg/ml KCN. 10% Digitonin w/v in Dimethyl sylphoxide
Coomassie Blue stain	1.5 g Coomassie blue, 550 ml ethanol, 110 ml acetic acid, 550 ml of dH_2O
Destain for Coomassie blue	10% acetic acid, 10% ethanol
Denaturing solution (Southern blots)	0.5 M NaOH, 1.5 M NaCl
DIG prehybridisation solution	5X SSC (see below), 1% Blocking reagent (Boehringer), 0.1% N-lauroylsarcosine, 0.02% SDS
DIG Buffer 1	0.1 M Maleic acid, 0.15 M NaCl, pH 7.5
DIG Buffer 2	Buffer 1 + 1% Blocking reagent
DIG Buffer 3	0.1 M Tris/HCl, 0.1 M NaCl, 50 mM $MgCl_2$, pH 9.5
DIG Washing buffer	DIG Buffer 1 + 0.3% Tween 20
Solution D	4 M guanidinium thiocyanate, 25 mM Na citrate (pH 7.0), 0.5% sarcosyl, 0.1 M 2-mercaptoethanol
Emetine	stored at 4°C as 100 mM stock solution in dH_2O
K-Buffer	50 mM KCl, 10 mM Tris/HCl (pH 8), 2.5 mM $MgCl_2$, 0.5% Tween 20, 100 µg/ml freshly dissolved Proteinase K
Lactate Buffer	46.5 ml of 0.1 M Tris HCl (pH 9.3), 3.5 ml of 30 mM NAD, 500 µl Hydrazine hydrate, 280 µl Lactate dehydrogenase (5mg/ml)
Lysing solution for blood	1% v/v Triton-X-100, 0.33 M sucrose, 5 mM $MgCl_2$, 10 mM Tris/HCl (pH 7.5)
Methylene blue stain	0.04% methylene blue, 0.5 M sodium acetate pH 5.2
MOPS	0.4 M MOPS, 3-[N-Morpholino]propane-sulphonic acid, 0.1 M Na acetate pH 7.0, 0.02 EDTA

MOPS gel	0.75 g agarose, 55.5 ml dH O, 7.5 ml 10X MOPS, 12 ml Formaldehyde when cooled to 60°C
Neutralisation Buffer (Southern blots)	1.5 M NaCl, 0.5 M Tris/HCl (pH 7), 1 mM EDTA
NET-High salt	1 M NaCl, 0.1 mM EDTA, 20 mM Tris/HCl (pH 8.0)
NET-Low salt	150 mM NaCl, 0.1 mM EDTA, 20 mM Tris/HCl (pH 8.0)
PBS (IOX)	0.026 M KCl, 0.15 M KH_2PO_4 (anhyd), 0.08 M N_2HPO_4, 1.37 M MaCl
Phosphate Buffer (KPi) 0.2 M	A-0.2 M KH_2PO_4; B - 0.2 K_2HPO_4. For 200 ml 56.5 ml of A and 43.5 ml of B were mixed and the volume adjusted with dH_2O (final pH 6.65)
Phosphate Buffer (KPi) 0.4 M	For pH 7.5: 16ml of A and 84ml of B diluted to 200ml. A-0.4 M ; B-o.4M. for 200ml 51 ml of A and 49ml of B (final pH 7.0).
Protein gel running buffer	15.2 g Tris, 72 g Glycine (pH 8.5), 0.1% SDS
Protein gel separating buffer	1.5 M Tris/HCl (pH 8.8)
Protein gel stacking buffer	0.5 M Tris/HCl (pH 6.8)
Pyruvate buffer	47.5 ml of 0.4 mM KPi (pH 7.0), 2.5 ml of 2 mM NADH, 28.7 µl Lactate dehydrogenase
RNA loading buffer	25% Ficoll, 0.25% bromophenol blue
SDS sample buffer (2X)	2.3% SDS, 3.5 mM Tris/HCl (pH 6.8),10% glycerol, 5% 2-mercaptoethanol, 0.25% bromophenol blue
Sucrose/Tris Buffer	0.25 M Sucrose, 0.01 M Tris/HCl (pH 7), 1 mM EDTA
Solution for Proteinase K (2X)	150 mM NaCl, 50 mM EDTA, 1% SDS
SSC (20X)	3 M NaCl, 300 mM Na citrate (pH 7.0)
TBE (10X)	89 mM Tris/HCl, 89 mM Boric acid, 2 mM EDTA
TE (100X)	1 M Tris /HCl (pH 8.0), 0.1 M EDTA

2:3 PREPARATION OF OLIGONUCLEOTIDES

Oligonucleotide primers were synthesised on an Applied Biosystems DNA synthesiser. To facilitate direct sequencing those 5' primers used to amplify products for subsequent sequencing had 2 Biotin moieties added at the 5' end during synthesis. The biotin phosphoramidite reagent was always used within 24 hours of opening.

Tables 2:1:1, 2:1:2 and 2:1:3 list the primers prepared for use in DNA amplification for sequencing.
Table 2:2 lists the primers used only for sequencing.
Table 2:3 lists sequencing primers which were gifts from Dr Ian Holt of the Department of Biochemical Medicine, University of Dundee.

2:4 PREPARATION OF DNA

2:4:1 Blood

Blood was collected into EDTA tubes and either processed immediately or stored at -20°C until DNA preparation. 50 µl of blood was mixed with 500 µl of TE buffer, vortexed briefly and centrifuged at 14000 *g*max for 5 minutes. The pellet was resuspended in a further 500 µl of TE and the process repeated twice. The final pellet was resuspended in 100 µl of K-buffer, vortexed and incubated at 56°C for 45 minutes. The protease was then inactivated by heating to 75°C for 10 minutes. The DNA prepared in this way was stored at -20°C until required.

2:4:2 Tissue culture cells

Cells from one 75 cm^2 flask (30 ml of 1 x 10^6/ml were washed once in PBS and the pellet resuspended in 10 times the cell pellet volume of PBS. Cells were lysed by adding 10% SDS to a final concentration of 0.05% and sterile NaCl was added to give a final concentration of 1M. This was mixed gently, then incubated overnight at 4°C to precipitate the chromatin. After centrifugation at 16000 *g*max for 30 minutes at 4°C, the supernatant was decanted and further SDS added to a final concentration of 0.05%.

Contaminating protein was removed by addition of 100 µg/ml Proteinase K. Digestion was effected at 65°C for 15 minutes. When the samples had cooled to room temperature they were extracted twice with phenol and once with chloroform/isoamylalcohol (24:1) once. The DNA was precipitated overnight at -20°C following addition of 1/10 volume of 3M NaAc (pH 5.0) and 2 volumes of

REFERENCE CODE	REGION OF MITOCHONDRIAL GENOME	SIZE OF FRAGMENT (np)
FR61/23B	D-LOOP	645
FR60/668L	D-LOOP	
FR14/618B	$tRNA_{phe}$	940
FR15/1570L	12S rRNA	
FR12/1444B	12S rRNA	680
FR13/2125L	16S rRNA	
FR10/2470B	16S rRNA	610
FR11/3080L	16S rRNA	
FR6/3013B	16S rRNA	690
FR7/3698L	ND1	
FR8/3628B	ND1	870
FR9/4505L	ND1	
FR16/4417B	$tRNA_{met}$	900
FR17/5258L	ND2	
FR25/5163B	ND2	627
FR26/5790L	$tRNA_{cys}$	
FR54/5703B	$tRNA_{asn}$	819
FR28/6522L	COI	

Table 2:1:1 Primers used to amplify PCR products for sequencing; region from np 23 in D-LOOP to np 6522 in COI gene. Reference code key: primers with -B suffix have two Biotin moieties at 5' end, number after dash is 5' np coordinate; primers with -L suffix are 3' primers on L-strand, number after dash is 3' np coordinate.

REFERENCE CODE	REGION OF MITOCHONDRIAL GENOME	SIZE OF FRAGMENT (np)
FR29/6456B	COI	796
FR30/7252L	COI	
FR31/7178B	COI	662
FR32/7840L	COII	
FR33/7773B	COII	789
FR34/8562L	ATPase 6	
FR35/8469B	ATPase 8	742
FR36/9211L	COIII	
FR37/9131B	ATPase 6	798
FR38/9937L	COIII	
FR39/9848B	COIII	825
FR40/10673L	ND4L	
FR41/10617B	ND4L	658
FR42/11275L	ND4	
FR43/11164B	ND4	750
L2/11914L	ND4	
FR51/11836B	ND4	717
FR44/12553L	ND5	

Table 2:1:2 Primers used to amplify PCR products for sequencing; region from np 6456 in COI gene to np 12553 in ND5 gene.

REFERENCE CODE	REGION OF MITOCHONDRIAL GENOME	SIZE OF FRAGMENT (np)
FR45/12472B	ND5	669
FR46/13141L	ND5	
FR1/12907B	ND5	827
FR2/13734L	ND5	
FR47/13627B	ND5	780
FR48/14447L	ND6	
FR55/14401B	ND6	866
FR23/15267L	Cyt *b*	
FR52/15194B	Cyt *b*	863
FR53/16057L	D-Loop	
FR63/15980B	$tRNA_{pro}$	643
FR62/54L	D-Loop	

Table 2:1:3 Primers used to amplify PCR products for sequencing; region from np 12472 in ND5 gene to np 54 in D-loop. Reference code key as in Table 2:1:1.

REFERENCE CODE	REGION
FR18/3519L	ND1
FR19/4171L	ND1
FR20/3321L	ND1
FR21/1268L	12S rRNA
FR22/5003L	ND2
FR24/14683H	tRNA Glu
FR56/8200L	COII
FR57/9656L	COIII
FR58/11548L	ND4
FR59/14070L	ND5
FR64/8630L	ATPase 6
FR65/10295L	ND3
FR66/12822L	ND5
FR67/10996L	ND4
FR68/1073L	12S rRNA
FR70/15217L	Cyt *b*
FR71/707L	12S rRNA
FR72/12080L	ND4
FR73/14576L	ND6
FR74/14827L	Cyt *b*
FR75/15715L	Cyt *b*
FR76/16208	D-loop
FR49/7324H	COI
FR50/7567L	tRNA ser
FR78/14973H	Cyt *b*

Table 2:2 Primers used as sequencing primers. Reference code key: primers with -H suffix encode the H-strand sequence, the number after the dash is the 5' np coordinate; primers with -L suffix encode the L-strand sequence, the number after the dash is the 3' np coordinate.

REFERENCE CODE	REGION OF MITOCHONDRIAL GENOME
IH3/4739H	ND2
IH5/16054H	D-loop
IH8/3122L	16S rRNA
IH14/3621L	ND1
IH27/15800L	Cyt *b*
IH28/483H	D-loop
IH30/2429H	16S rRNA
IH31/2363L	16S rRNA
IH33/5914L	COI
IH42/15460H	Cyt *b*
IH44/13523H	ND5
IH60/6880L	COI
IH63/7935L	COII
IH91/3100H	16S rRNA

Table 2:3 Primers used as sequencing primers; a gift from Dr Ian Holt, Department of Biochemical Medicine, University of Dundee.

absolute ethanol. The DNA was pelleted by centrifugation at 14000 g max for 10 minutes; washed in 70% ethanol and dried at room temperature. RNA contaminating the pellet was removed by adding boiled RNase to a concentration of 100 µg/ml and incubating at 37°C for 1 hour. SDS and Proteinase K treatment was repeated as above followed by extraction with phenol, extraction with chloroform and precipitation with ethanol. The final, dried DNA sample was resuspended in 100 µl TE and stored at -20°C.

2:4:3 Buccal cells

10 ml of sterile distilled water was swilled round the mouth for approximately one minute before being put into a 15 ml falcon tube. After centrifugation at 3000 g max for 5 minutes the buccal cell pellet was resuspended in 500 µl TE and treated as for blood (section 2:4:1).

2:5 ESTIMATION OF DNA CONCENTRATION USING ETHIDIUM BROMIDE PLATES

In order to estimate the amount of DNA extracted from either blood or cells 20 ml of 1% agarose was prepared: to this 1 µl of 10 mg/ml of ethidium bromide was added, giving a final concentration of 500 ng/ml. This was poured into a standard (90 mm) petri dish and allowed to set. A dilution series from undiluted to 1:10,000 of a known concentration of DNA was spotted onto the agarose alongside a dilution series of prepared DNA samples. Once dry the plate was viewed under a UV light and the amount of DNA estimated by comparing the intensity of fluorescence.

2:6 POLYMERASE CHAIN REACTION (PCR)

2:6:1 PCR reaction conditions

Twenty microlitre reactions were set up; in each approximately 100 ng of template DNA was mixed with 200 µM each of dATP, dCTP, dTTP and dGTP and 20 pmol each of forward and reverse primers in 1X Taq polymerase buffer supplied by the manufacturer of the Taq polymerase. One unit of Taq polymerase was added and the reaction overlaid with mineral oil before thermal cycling.

2:6:2 Conditions for thermal cycling

A Cambio thermal cycler was employed. Unless otherwise stated, the conditions for amplification were:

94°C x 6 minutes		
55°C x 1 minute	}	
72°C x 3 minutes	}	30 cycles
94°C x 1 minute	}	
72°C x 15 minutes		

In order the minimise the risk of amplification artifacts being mistaken for sequence mutations, when amplification was carried out for sequencing four reaction were set up and the amplified products pooled before purification. Amplified products were always checked by agarose gel electrophoresis and subsequent ethidium bromide staining.

2:7 PURIFICATION OF AMPLIFIED PRODUCTS

Where the agarose gel indicated that there was only one major amplified product, this was purified using the PROMEGA "Magic" PCR purification system or the Qiagen PCR purification system. Where there were minor products in addition to the obvious major product, the major product was purified using NA45 paper.

2:7:1 Activation of NA45 paper

The paper was washed at room temperature for 10 minutes in 10 mM EDTA, then 5 minutes in 0.5 M NaOH. After several rapid washes in sterile distilled water the activated paper can be stored in distilled water at 4°C for several months.

2:7:2 Separation of amplified product

The amplified product was loaded, alongside size markers, onto 1% agarose gel and run overnight at 20 volts to ensure good separation of bands. After ethidium bromide staining the gel was viewed under UV light and a cut, the width of the well, made just beyond the band to be isolated. A piece of activated paper was inserted into the cut and the gel electrophoresed for a further 10 minutes at 100 volts. The NA45 paper was removed and viewed under UV to check that the DNA had run into the paper.

2:7:3 Elution of amplified product

The DNA-containing paper was washed three times in 500 µl Low salt NET, followed by incubation in High salt NET at 68°C for 15 minutes. The eluate was removed and stored, while a further 100 µl of High salt NET was added and the paper further incubated at 68°C for 30 minutes. The 2 eluates were pooled and

precipitated by addition of 1/10 volume of 3M Na acetate and 2 volumes of ethanol. The purified product was stored at -20°C in this form.

2:8 RESTRICTION ENZYME DIGESTION

2:8:1 Total cell DNA

DNA was prepared as described in Section 2:4:1. 10 µg was digested with 10 units of restruction enzyme, according to the manufacturers instructions, in a total volume of 200 µl. Digestion products were precipated in ethanol and after drying and resuspension in TE electrophoresed through 0.8% agarose gels at 100 volts for 2 hours. DNA was visualised by Ethidium bromide staining.

2:8:2 Amplified PCR products

PCR was carried out with appropriate forward and reverse primers to amplify specific mitochondrial DNA regions. After purification, 1 µg of amplified product was digested with 1 unit of restriction enzyme according to the manufacturers instructions. Digestion products were electrophoresed through 1.5% agarose or 3% Nusieve agarose gels at 100 volts for 2 hours. DNA was visualised by Ethidium bromide staining.

2:9 DNA STRAND SEPARATION PRIOR TO SEQUENCING

The 2 strands of purified amplified products were separated using Streptavadin-coated Dynabeads (see Figure 2:1). For each 50 µl of purified product 25 µl of Dynabeads were washed once with 1X B&W Buffer, resuspended in 50 µl of 2X B&W Buffer and mixed with 50 µl of purified PCR product. The DNAwas bound to the Streptavidin-coated beads (via the biotin) during incubation at room temperature for 15 minutes after which the beads were washed once with 1X B&W Buffer. In order to elute the non-biotinylated strand the beads were resuspended in 8 µl of freshly prepared 0.1 M NaOH and kept at room temperature for 10 minutes.

After this period the non-biotinylated strand was removed in the 8 µl to a fresh tube and 4 µl of 0.2 M HCl plus 1 µl of 1 M TrisHCl (pH 7.5) added to neutralise. The volume was made up to 20 µl with distilled water and the DNA precipitated at -20°C using 3 M Na acetate and ethanol. The Dynabeads bearing the biotinylated strand were washed successively with 50 µl 0.1 M NaOH, 50 µl 1X B&W Buffer, and 50 µl TE Buffer. The beads were finally resuspended in 7 µl of sterile distilled water, ready for sequencing.

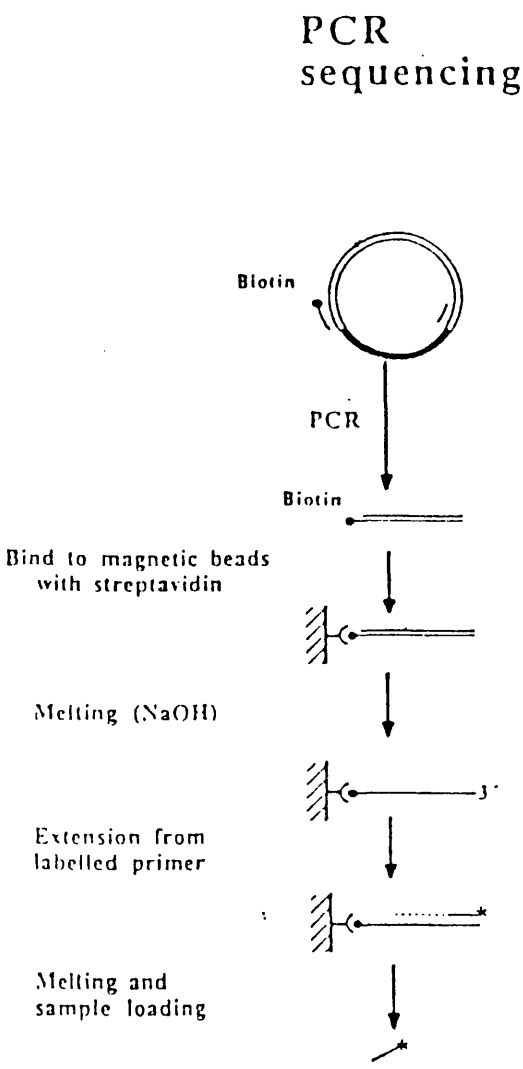


Figure 2:1 PCR sequencing using a biotinylated primer and Streptavidin-coated Dynabeads. Taken from Hultman et al, 1989.

2:10 SEQUENCING OF AMPLIFIED PRODUCT

2:10:1 Biotinylated strand

Annealing reaction:
7 µl Dynabeads with +Bio strand attached
2 µl Sequenase dilution buffer
1 µl Primer (1 pmol)
Incubated at 65°C for 2 minutes, allowed to cool slowly to 37°C then put at 4°C for up to 4 hours before sequencing.

Sequencing was then carried out using ^{35}S dATP according to the Sequenase Version 2 protocol (US Biochemicals). Before addition of stop solution the supernatant containing excess nucleotides and primers was taken from the beads using a magnet. The sequenced products were then eluted off the beads by addition of stop solution. It was not necessary to remove the beads before loading samples onto the sequencing gel.

2:10:2 Sequencing the non-biotinylated strand

The non-biotinylated strand was precipitated at -20°C for at least 24 hours. After washing in 70% ethanol and drying at room temperature the precipitated -Bio strand was taken up in 7 µl of distilled water and sequenced exactly according to the Sequenase Version 2 protocol (US Biochemicals).

2:10:3 Electrophoresis of sequenced products

Sequencing gels were made using Long Ranger gel solution and 0.6 x TBE used as running buffer. A BRL sequencing gel apparatus was used. After denaturation at > 80°C for 3 minutes 2 µl of +Bio samples, or 3 µl of -Bio samples were loaded and electrophoresed at constant power (60 Watts) for the required length of time. The polyacrylamide gel was transferred to 3 MM paper (Whatman), protected by Saran wrap and dried using a Genevac pump on a BioRad gel drier (model 583). The dried gel was exposed to X-ray film at room temperature and the film subsequently developed using an X-ray processer (X-o-graph, Compact x 2).

2:11 SINGLE-STRANDED CONFORMATIONAL POLYMORPHISM (SSCP) ANALYSIS

2:11:1 Denaturation of samples

To 5 µl of purified amplified product 1 µl of 0.5 MNaOH/10 mM EDTA was added and samples were incubated at 42°C for 5 minutes after which 2.5 µl of loading buffer were added to each.

2:11:2 Electrophoresis of samples

Samples were loaded onto a 16 cm x 11 cm MDE gel, prepared according to the manufacturer's instructions, after each well had been washed out with 1X TBE. Electrophoresis was carried out at constant power (20 Watts) with circulating water to maintain the gel temperature around 20°C. Each gel was run until the xylene cyanol dye had reached the bottom of the gel, normally approximately 4 hours.

2:11:3 Staining of gels

Electrophoresed samples in MDE gels were stained using silver stain according to the manufacturer's instructions. The silver stain kit and protocol was supplied by BioRad. Briefly, the gel was agitated sequentially with:

40% Methanol	-	30 minutes
10% Ethanol	-	2 x 5 minutes
Oxidiser	-	5 minutes
dH_20	-	4 x 5 minutes or until yellow colour faint
Silver solution	-	20 minutes
dH_20	-	1 minute
Developer	-	30 seconds; 2 x 5 minutes
5% Acetic acid	-	overnight

The stained gel was transferred to 3 MM paper and dried using a BioRad gel drier.

2:12 SOUTHERN BLOTTING

2:12:1 Transfer of DNA to membrane

DNA digested with an appropriate restriction enzyme was electrophoresed through agarose gels, denatured by soaking for 30 minutes in denaturing solution and neutralised by soaking for 30 minutes in neutralising solution, before being transferred to positively charged nylon membrane (Boehringer Mannheim) by capillary blotting overnight in 6 x SSC. DNA was subsequently fixed to the membrane by baking at 80°C for 120 minutes.

2:12:2 DIG-random labelling

1 µl of denatured, purified PCR product was labelled by incubation with 2 µl of dNTP labelling mixture (1Ox) containing DIG-11-dUTP, 2ul of hexanucleotide mixture and 1 µl (2u/µl) of Klenow enzyme in a total volume of 20 µl. Afteri overnightincubation at 37 C the reaction was terminated by addition of 2 µl of 200 mM EDTA. The labelled DNA was precipitated by incubation with 0.1 volume of 4 M LiCl and 3 volumes of ethanol at -20°C.

2:12:3 Hybridisation

Membranes were prehybridised in DIG-prehybridisation solution at 42°C for at least 2 hours. 200 ng of denatured DIG-random-labelled PCR product was added into the prehybridisation solution and hybridisation carried out at 42°C overnight. The blot was washed twice in 2 x SSC/0.1% SDS at room temperature and twice in 0.1 x SSC/0.1% SDS at 65°C .

2:12:4 Detection of hybridised molecules

Hybridisation was detected according to Boehringer Mannheim's DIG protocol. After washing, the membrane was equilibrated in DIG Buffer 1 for 5 minutes. The membrane was then blocked by gentle agitation in DIG Buffer 2 for 30 minutes. The membrane was then incubated in anti-DIG-alkaline phosphatase at 1:10,000 (75 mu/ml) for 30 minutes with gentle agitation. The membrane was then washed in DIG-Washing buffer for at least 30 minutes after which it was equilibrated in DIG Buffer 3 for 5 minutes before addition of substrate AMPPD (4-methoxy-4-(3-phosphate-phenyl)-spiro(1,2-dioxetene-3,2-adamantane) disodium salt) solution (10 mg/ml). After incubation for 5 minutes the membrane was removed (the substrate solution can be reused) and blotted for a few seconds on Whatman 3 MM paper. The membrane was then sealed in a

plastic bag and incubated at 37°C for 10-15 minutes before exposure to X-ray film for 15-25 minutes at room temperature.

2:13 Culture of Epstein-Barr Virus (EBV)-transformed cell-lines

Transformation of lymphocytes by Epstein-Barr Virus was carried out at the European Collection of Animal Cell Cu;tures (ECACC), or at the Department of Medical Genetics, Yorkhill Hospital, Glasgow. Cells were received as frozen vials or as growing, suspension cultures. Cells were maintained in RPMI + 10% heat inactivated foetal calf serum + 2 mM Glutamine + 100 u/ml Penicillin + 100 µg/ml Streptomycin. Growth conditions were 37°C in humidified 5% CO_2.

2:14 RNA MANIPULATIONS

2:14:1 Preparation of RNA from tissue-culture cells

5 x 10^9 cells were resuspended in 2 ml of solution D. Sequentially added were 200 µl of 2 M Na acetate (pH 5), 2 ml of water equilibrated Phenol, 400 µl of chloroform/isoamyl alcohol (24:1), mixing after each addition.

After shaking for 10 seconds this mixture was kept on ice for 15 minutes, then centrifuged at 10,000 *g*max for 20 minutes at 4°C. The aqueous phase was removed to a fresh tube and 2 ml of isopropanol added to precipitate the RNA at -20°C. After 24 hours the precipitate was spun down at 10,000 *g*max for 20 minutes at 4°C. After centrifugation RNA was present in the aqueous phase which was transferred to a fresh tube, mixed with 1 ml of isopropanol and put at -20°C for at least one hour. After centrifugation at 10,000 *g*max for 20 minutes the RNA pellet was dissolved in 300 µl of solution D and 300 µl of isopropanol was added to reprecipitate the RNA at -20°C overnight. After washing the pelleted RNA in 70% ethanol and drying, the RNA was taken up in 50 µl TE and stored at -70°C until required.

2:14:2 Electrophoresis of RNA

To 10 µg of RNA were added 10 µl of deionised formamide, 2 µl of 10 x MOPS and 3.2 µl of formaldehyde. The solution was incubated at 65°C for 15 minutes then stored on ice until ready to load into the gel. 5 µl of loading buffer was added. Samples were electrophoresed through 1% agarose gel containing formaldehyde (see MOPS-gel) gel at 80 Volts for one hour.

2:15 NORTHERN BLOTTING

2:15:1 RNA transfer

RNA was transferred to Boehringer positively charged nylon membrane by overnight capillary blotting of the gel in 10 x SSC.

After fixation of RNA to the membrane, by baking at 80°C for 2 hours or 120°C for 30 minutes, successful transfer was confirmed by methylene blue staining of membrane, as follows: Briefly: the membrane was soaked in methylene blue stain for 10 minutes, then destained with a few changes of H_2O until very little blue background remained.

2:15:2 Northern Hybridisation

Hybridisation was carried out in hybridisation bottles with a mock filter of the same dimensions as the test filter separated from the test filter by nylon mesh. Prehybridisation was carried out at 50°C for > 4 hours using DIG prehybridisation solution. 200 ng of denatured, DIG-labelled PCR product was added and hybridised overnight at 50°C. Washing and detection was as for Southern blots.

2:16 ANALYSIS OF MITOCHONDRIAL TRANSLATION PRODUCTS

2:16:1 ^{35}S Methionine labelling

1 x 10^6 cells were incubated in 100 µl of RPMI medium minus Methionine for one hour at 37°C. Emetine was added at concentrations of 100 nM or 10 nM, in the presence and absence of 1 mM chloramphenicol. 25 µci of ^{35}S labelled methionine (1175 Ci/mmol) was then added and the cells incubated at 37°C for a further 1 hour. Cells were then washed in 1 x PBS and resuspended in 20 µl of 1 x SDS-loading buffer.

2:16:2 Electrophoresis of proteins

After denaturation at 100°C for 10 minutes samples were electrophoresed through an SDS/12% polyacrylamide gel at constant current (20 mA) for 4 hours. Gels were stained with Coomassie blue, and destained overnight before being washed in "Amplify" (Amersham) for 30 minutes. After drying onto 3 MM paper proteins were visualised fluorographically by exposing to film at -70°C for at least 24 hours before developing.

2:17 CYTOCHROME-*c* OXIDASE (COX) ASSAY

5 x 10^6 cells were washed with 1 x PBS and resuspended in 1 ml of Sucrose-Tris (pH 6.7). These were kept on ice until reagents prepared. Immediately prior to assay the cells were disrupted by being frozen in dry ice/ethanol and thawed at 37°C three times. 30 µl of disrupted cells were mixed with 1.8 µl of 10% Digitonin for 10 minutes on ice. 25 µl of this was mixed (in a 1 ml cuvette) with 470 µl of KPi buffer and 500 µl of cytochrome *c* which had been fully reduced by adding Na dithionite. Absorbance at 550 nm was read over a period of 5 minutes at room temperature. In order to measure the level of autoxidation (it should be minimal) a reference sample was set up with no cells but with 10 µl of 6.5 mg/ml KCN. After 30 seconds at room temperature the absorbance at 550 nm was measured. If any rate was observed the cytochrome *c* had not been fully reduced and further Na dithionite was added until no rate was observed.

2:18 SUCCINATE/CYTOCHROME *c* REDUCTASE (SCCR) ASSAY

Cells disrupted for COX assay were also used in the SCCR assay but without Digitonin treatment. Mixed in a cuvette were: 800 µl of 30mM KPi Buffer (pH 7.5) 50 µl of 60 mM Succinate, 50 µl of 2 mM Cytochrome *c* which was fully oxidised i.e. no Na dithionite added, 50 µl of 50 mg/ml BSA, 50 µl of 20 mM KCN and 25 µl disrupted cells.

Absorbance at 550 nm was measured at room temperature over a period of 5 minutes against a blank with every component except cells.

2:19 LACTATE/PYRUVATE MEASUREMENTS

2:19:1 Preparation of cells

5 x 10^5 cells were resuspended in 2 ml of RPMI 1640 (from ICN) + 4% foetal calf serum + 2 mM glutamine + 50 µg/ml uridine and incubated in 25 cm^2 flasks for 48 hours at 37°C in humidified 5% CO_2.

After 48 hours the medium was removed and 1/5 volume of 10% TCA added before centrifugation at 3000 g max for 10 minutes. This was frozen at -20°C overnight until ready to assay. Immediately prior to assay samples were centrifuged at 3000 *g*max for 5 minutes and the supernatant assayed for both lactate and pyruvate.

2:19:2 Lactate assay

Standards within the range 100 μM-20 mM lactic acid were prepared. 25 μl of substrate was added to 975 μl lactate buffer and incubated at 37°C for approximately 1 hour. Absorbance of NADH at 340 nm was measured for each against a reference with lactate buffer and water.

2:19:3 Pyruvate assay

Standards within the range 1-200 μM sodium pyruvate were prepared. 200 μl of substrate was added to 800 μl pyruvate buffer and incubated at 37°C for approximately 30 minutes before measuring optical density at 340 nm.

2:20 MTT ASSAYS

2.0×10^6 cells were incubated in 25 ml of RPMI medium in 25 cm^2 flasks. 500 μl samples were taken in triplicate and 50 μl of MTT (3-(4,5-dimethyj-thiazol-2-yl)-2.5-di-phenyl tetrazolium bromide) at 5 mg/ml added to each. After incubation at 37°C in humidified 5% CO_2 for 1 hour 500 μl of 10% SDS was added to each sample which was re-incubated at 37°C for up to 24 hours. Optical density at 570 nm was measured on all samples at the end of the sampling period.

2:21 AUDIOMETRY

Pure tone audiometry was performed (by staff of the ENT Dept., Victoria Infirmary, Glasgow) in an acoustically treated booth to standard ISO/DIS 8253, using a Kamplex AC3 clinical audiometer.

CHAPTER 3 INVESTIGATION OF PATIENTS SUFFERING FROM NON-SYNDROMIC SENSORINEURAL HEARING LOSS

3:1 STUDIES OF PATIENTS WITH SENSORINEURAL HEARING LOSS OF UNKNOWN AETIOLOGY

Sensorineural hearing loss is one of the most common symptoms reported in diseases associated with mutations of mitochondrial DNA. The identification of a patient with sensorineural hearing loss in addition to LHON (further discussed in Chapter 5) prompted this investigation of patients with no known aetiology for their deafness. The intention was to examine mitochondrial DNA from patients with non-syndromic deafness for readily identified mutations. This was to be done initially by means of PCR and restriction digestion of amplified products.

3:1:1 Selection of patients

This study was undertaken in collaboration with Mr Guy Vernham at the Victoria Infirmary, Glasgow. He selected patients suffering from sensorineural hearing loss, with no known aetiology, who fulfilled specific criteria. The age range was from 10 years to 55 years, in order to exclude a) early childhood deafness which can have many causes and b) presbycusis (deafness due to advanced age). Patients must not have been exposed to an environment of extreme or prolonged noise; in addition patients should at no time in their lives have had a course of aminoglycoside antibiotics.

Mr Vernham measured the degree of bilateral hearing loss by means of pure-tone audiometry, as described in Section 2:21 and defined the deafness as mild, moderate or severe. The definitions of 20-35 dB as mild, 35-85 dB as moderate and > 85 dB as severe were made by Mr Vernham, with the additional factor of the age of the subject taken into account. Detailed audiometric data was not made available to us, consequently the precise degree of deafness of each patient is not given. 33 patients were examined; 27 age, sex and ethnically matched normal individuals were selected by Mr Vernham and examined audiometrically before inclusion in the study as controls. Blood samples were taken from all patients and controls and processed immediately or stored at -20°C until DNA extraction as in Section 2:4.

3:1:2 Medical history of patients obtained by questionnaire

In order to identify patients who had other symptoms commonly reported in mitochondrial disease e.g. muscle weakness, in addition to their hearing loss, a questionnaire was produced. This was completed by all patients and provided information regarding the family history of deafness or other reported symptoms.

Each patient was asked to complete the questionnaire as shown in Figure 3:1. Age of onset of deafness was not known in the majority of cases; no patient reported muscle weakness or epilepsy. The only eye disorder identified was short or long-sightedness with a requirement for spectacles. One patient, number 112, had a history of diabetes which was said to be "borderline" (her words), no other clinical information regarding her diabetes was available. Of the 33 deaf patients, 6 identified migraine as a problem (5 females, 1 male); this is very similar to the incidence of migraine reported by the 27 normal controls (18.5% c.f.19%).

These figures are both much higher than the normally quoted incidence of 2% of the general population (C Reid, personal communication). The difference is probably due to misunderstanding by the patients and controls questioned in our study, of what constitutes a migraine headache. Figures gathered by medical audit are calculated from numbers of patients treated for migraine. Many people use the term "migraine" when what they mean is a bad headache. The data from the questionnaires indicates that the patients in this study suffered from nothing other than sensorineural hearing loss, indicative of their diagnosis as having non-syndromic deafness.

3:1:3 Restriction fragment length polymorphism (RFLP) analysis

A number of restriction enzyme sites, mutations at which had previously been associated with various mitochondrial diseases, were screened in both patients and controls. As shown in Table 3:1 sites investigated were np 3243, mutations at which are associated with MELAS and identified in a number of patients with diabetes associated with deafness; np 3460; np 4917, np 7440, np 11777, and np 13708, all of which have been found in association with LHON. All of the sites investigated have previously been associated with phenotypes involving sensorineural loss of function; hearing loss in np 3243 and visual loss in LHON. Table 3:1 shows the primers used to amplify, by PCR, segments of mitochondrial DNA containing the restriction enzyme sites. Table 3:2 shows the results of the RFLP analysis for the 33 patients in the study. The only difference from wild type found was in patient number 31 who had lost the *Xba*I site at 7440. This patient was further investigated and the results are given in Chapter 4. No

Would you please complete the following questionnaire and return it to:

Mrs Fiona Reid
Robertson Institute of Biotechnology
Dumbarton Road
Glasgow G12 8QQ

NAME: .....

ADDRESS: .....

DATE OF BIRTH: .....

NATIONALITY: .....

DO YOU OR HAVE YOU EVER SMOKED?.....

AGE AT ONSET OF DEAFNESS: .....

HAVE YOU ANY HISTORY OF:

1. Diabetes .....
2. Migraine .....
3. Muscle Weakness .....
4. Visual Disorders .....
5. Epilepsy .....

Please gives names and dates of birth of any brothers or sisters (living or dead). Include half brothers or sisters and state which parent you have in common.

.....

.....

How many brothers and sisters (living or dead) does your Mother have?

.....

How many brothers and sisters (living or dead) does your Father have?

.....

Have any of these relatives suffered from any of the above disorders, including deafness?

.....

If any relatives are affected in any way could you supply their name and address?

Figure 3:1 Questionnaire completed by patients with non-syndromic hearing loss of no known aetiology, and control individuals.

							PREDICTED DIGESTION PRODUCTS (np)	
FORWARD PRIMER	REVERSE PRIMER	SIZE OF PRODUCT (np)	RESTRICTION ENZYME SITE (np)	LOSS OR GAIN	ENZYME	DISEASE	NORMAL	MUTANT
FR6/3013B	FR7/3698L	685	3243	GAIN	*Apa*I	MELAS	685	455/230
FR6/3013B	FR7/3698L	685	3460	LOSS	*Bst*HI*	LHON	238/447	685
FR16/4417B	FR17/5258L	841	4917	GAIN	*Mae*I	LHON	345/204/149/90 /38	303/204/149/90 /38
FR31/7178B	FR32/7840L	662	7440	LOSS	*Xba*I	LHON	400/262	662
L1/11688H	L2/11914L	226	11777	LOSS	*Sfa*NI	LHON	137/89	226
FR1/12907B	FR48/14447L	1540	13705	LOSS	*Bst*NI	LHON	798/742	1540

Table 3:1 Primers used to amplify PCR products containing specific restriction enzyme sites. Forward primers are those which anneal to the 5' end of the sequence to be amplified, reverse primers anneal to the 3' end.

* This is an isochizomer of *Aha*II

					RFLP Analysis					
No	Age	Sex	Age of Onset	Enzyme: Site:	ApaI 3243	AhaII 3460	MaeI 4917	*Xba*I 7440	SfaNI 11778	BstNI 13708
1	30	F	Nk		+	+	+	+	+	+
2	31	F	Nk		+	+	+	nd	+	nd
3	38	M	Nk		+	+	+	nd	+	+
4	24	F	Nk		+	+	nd	+	+	nd
13	39	M	Nk		+	+	nd	+	+	nd
14	28	F	27		+	+	nd	+	+	+
15	35	M	Nk		+	+	nd	+	+	nd
16	27	F	Nk		+	+	nd	+	+	+
17	36	F	< 12		+	+	nd	+	+	nd
18	25	M	Nk		+	+	nd	nd	+	nd
21	28	F	25		+	+	nd	nd	+	nd
25	40	F	20		+	+	nd	nd	+	nd
*31	11	F	3		+	+	+	-	+	+
34	56	F	49		+	+	+	+	+	+
35	35	F	Nk		+	+	+	+	+	+
36	27	F	Nk		+	+	+	+	+	+
37	44	M	Nk		nd	nd	nd	+	+	+
38	48	F	Nk		nd	nd	nd	+	+	nd
39	32	F	Nk		+	+	nd	+	+	nd
41	46	F	Nk		+	+	nd	+	+	nd
42	55	M	Nk		+	+	+	+	+	nd
44	48	M	Nk		+	+	+	+	+	nd
45	24	M	Nk		+	+	+	+	+	nd
46	51	M	Nk		+	nd	nd	+	nd	nd
=47	12	M	Nk		+	nd	+	+	nd	nd
=48	11	M	Nk		+	+	+	nd	nd	nd
49	42	F	40		+	nd	nd	+	nd	nd
50	38	M	Nk		+	nd	nd	+	nd	nd
51	42	F	35		+	nd	nd	+	nd	nd
52	42	F	40		+	nd	nd	+	nd	nd
80	28	M	22		+	nd	nd	+	+	+
95	50	F	49		+	nd	nd	+	+	+
112	40	F	30		+	+	+	+	+	+

Table 3:2 Compilation of RFLP analysis of patients with non syndromic sensorineural hearing loss

* Index case in pedigree study - see Chapter 4
= Brothers
Nk Not known
nd Not determined
\+ Wild type
\- Mutant

deviation from normal was found at any site tested in any of the 27 normal controls screened.

3:1:4 Analysis of the *Apa*I restriction site encompassing np 3243

Creation of an *Apa*I restriction site by a nucleotide change at np 3243 is found in patients suffering from MELAS or in some cases of diabetes with deafness (see Section 1:5), due to an A->G transition in the $tRNA_{Leu(UUR)}$ gene.

The mechanism by which this mutation results in a pathological phenotype remains unknown. The possibility of involvement of this mutation in the development of non-syndromic deafness prompted us to screen our patients for this mitochondrial DNA mutation.

None of the deaf patients screened had gained an *Apa*I site in the segment of DNA spanning np 3243. Figure 3:2A shows a typical RFLP analysis using *Apa*I on two of the patients: numbers 3 and 17. As positive controls DNA was also analysed from cybrid cell lines produced from fusion of rho° cells and cells derived from MELAS patients with varying proportions of mutant mitochondrial DNA. These cybrids maintained a stable percentage of mutant DNA (Ian Holt, personal communication). Cell-line 206.3243 (143B - derived) had previously been shown to have > 90% mutant mitochondrial DNA, whilst B2.3243 (A549 - derived) had approximately 45% mutant. Without densitometry these figures cannot be accurately confirmed, but examination of Figure 3:2B indicates that our restriction digestion experiments gave approximately similar results. This suggests that the lack of digestion product in the patient samples is not simply due to the enzyme being inactive.

3:1:5 Evaluation of the presence of deletions in the mitochondrial DNA of deaf patients using PCR

As outlined in detail in Chapter 1:8, rearrangements of mitochondrial DNA have been identified in a number of mitochondrial diseases in which sensorineural deafness is a feature. For this reason it was decided to screen as many deaf patients as possible for the presence of deletions or duplications of mitochondrial DNA. Constraints of time allowed only a limited study, the results of which are presented below.

Patients 1, 36, 50 and 80 were screened for the presence of partially deleted mitochondrial DNA molecules using forward and reverse primers which, in theory, would normally amplify large sections of mt DNA, but which will

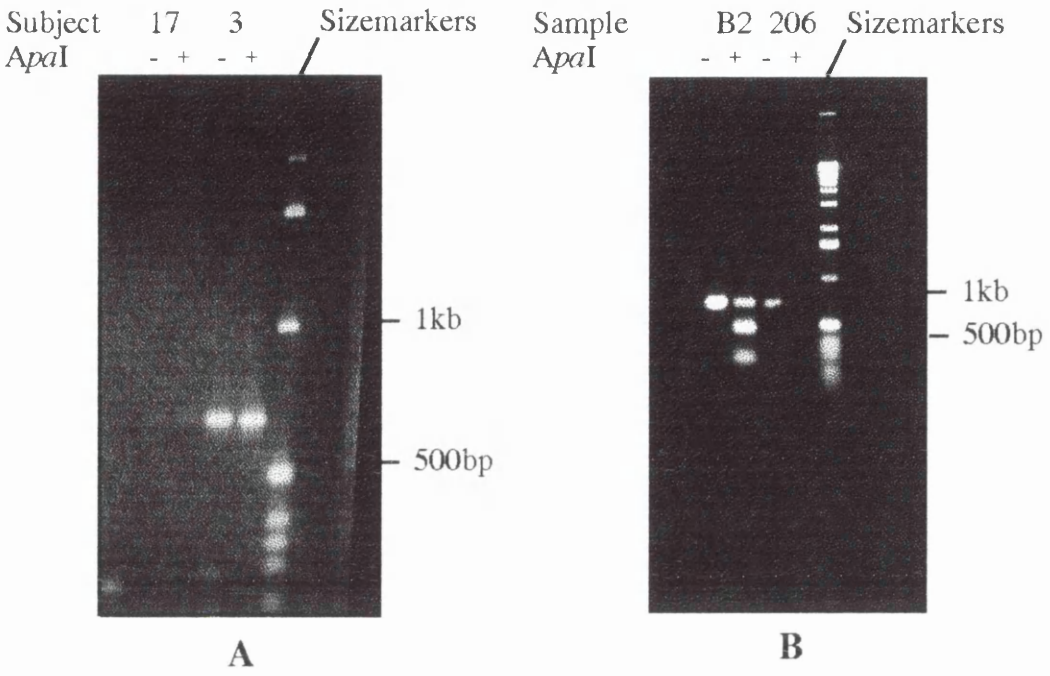


Figure 3:2 **A** RFLP analysis of the genotype at np 3243 by restriction digestion of a 690 np PCR product created using FR6/3103B as forward primer and FR7/3698L as reverse primer. In both patients, 17 and 3, there has been no gain of an A*pa*I site; the 690 np product remains uncut.

B Positive controls to show that the A*pa*I enzyme is effective. PCR product created using template DNA from cybrid cell-lines with varying proportions of the mutation at np 3243 were cut with A *pa*I. The digestion results are in agreement with previous estimates that B2.3243 cells have 45% mutant and 206.3243 cells 90% mutant mitochondrial DNA.

These experiments were carried out on different days.

produce much smaller sized PCR products in the presence of partially deleted template DNA. Contained in Table 3:3 are details of the primers used and sizes of products expected if one of the previously identified deletions given is present. Figure 3:3 is an illustration of the human mitochondrial genome showing the position of the three deletions described in Table 3:3 in relation to the pairs of oligonucleotide primers used in PCR reactions. I had previously shown that under the conditions for PCR used products greater than 3.5 kb cannot be amplified.

Figure 3:4 shows the products obtained using DNA from patient 1 as template, showing no evidence of a large scale deletion. Similar results were obtained with patients 36, 50 and 80.

Figure 3:5 is a diagramatic representation of the expected PCR product with each pair of primers in the presence of each deletion. It should be noted that under the PCR conditions used here deletions B & C would give the same result. Only if PCR conditions which allow amplification of greater than 5 kb were developed could the two be distinguished.

3:1:6 Evaluation of the presence of duplications/deletions by Southern analysis of digested mitochondrial DNA

DNA from a small number of patients was digested using *Pvu*II. Normal human mitochondrial DNA has only one restriction site for the enzyme *Pvu*II, consequently restriction digestion of mitochondrial DNA with this enzyme linearises the DNA molecule. After Southern transfer the DNA was probed using a random DIG-labelled PCR product created using FR31/7178B as forward primer and FR32/7840L as reverse primer.

This probe should hybridise to the COI gene which lies outwith the most common deletion found in mitochondrial disease, the breakpoints of which map to np 8470 and np 13459. In normal linearised mitochondrial DNA this probe should hybridise and be visualised as a 16.5 kb band. If the common deletion is present then the probe will still be able to hybridise and reveal a much shorter DNA molecule. Obviously if a deletion is present which encompasses the region to which the probe will hybridise, no mitochondrial DNA molecule can be visualised.

Figure 3:6 is a Southern blot showing DNA treated in this way from patients, all of whom have a single band of approximately 16 kb, indicating that no large scale

Primer Pair	Forward Primer	Reverse Primer	Size of product from mitochondrial DNA: Wild Type	A	B	C
1	FR1/12907B	FR2/1373L	827	-	-	-
2	FR8/3628B	FR9/4505L	877	680	877	877
3	FR8/3628B	FR23/15267L	584	-	584	584
4	FR24/14683H	FR2/1373L	10106	-	-	5117
5	FR8/3628B	FR23/15267L	11639	1070	5339	6375

Table 3:3 PCR amplification products expected with distant forward and reverse primers. The deletion break points previously reported are:

A - Deletion identified in Diabetes + Deafness - np 4308-14874 (Ballinger et al, 1992)
B - Deletion identified in Parkinson's disease - np 7900-14200 (Ikabe et al, 1990)
C - Deletion identified in KSS - np 8470-13459 (Shoffner et al, 1989)

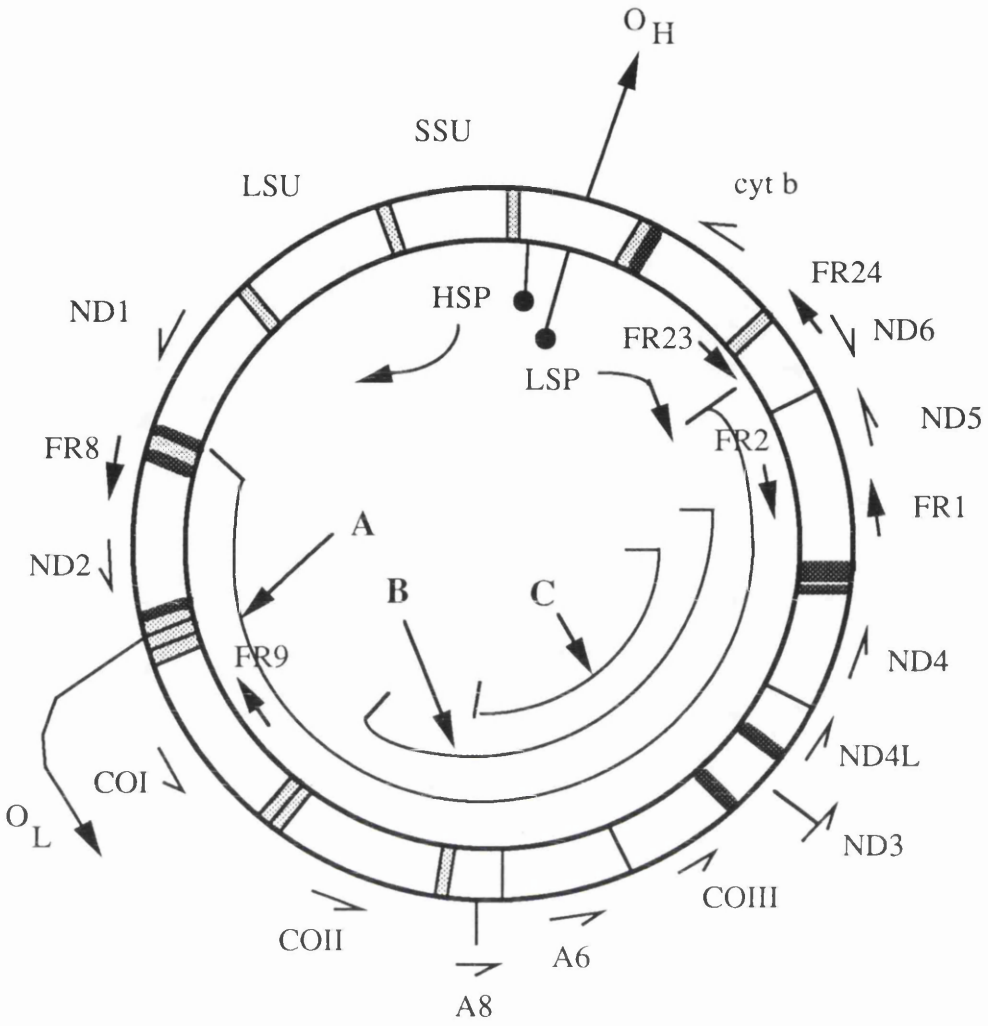


Figure 3:3 Illustration of human mitochondrial genome showing the PCR primers described in Table 3:3 in relation to the position of previously descibed deletions A,B and C.

Primers are abbreviated :

FR1 = FR1/12907B;	FR2 = FR2/1373L
FR8 = FR8/3628B;	FR9 = FR9/4505L
FR24 = FR24/14683H;	FR23 = FR23/15267L

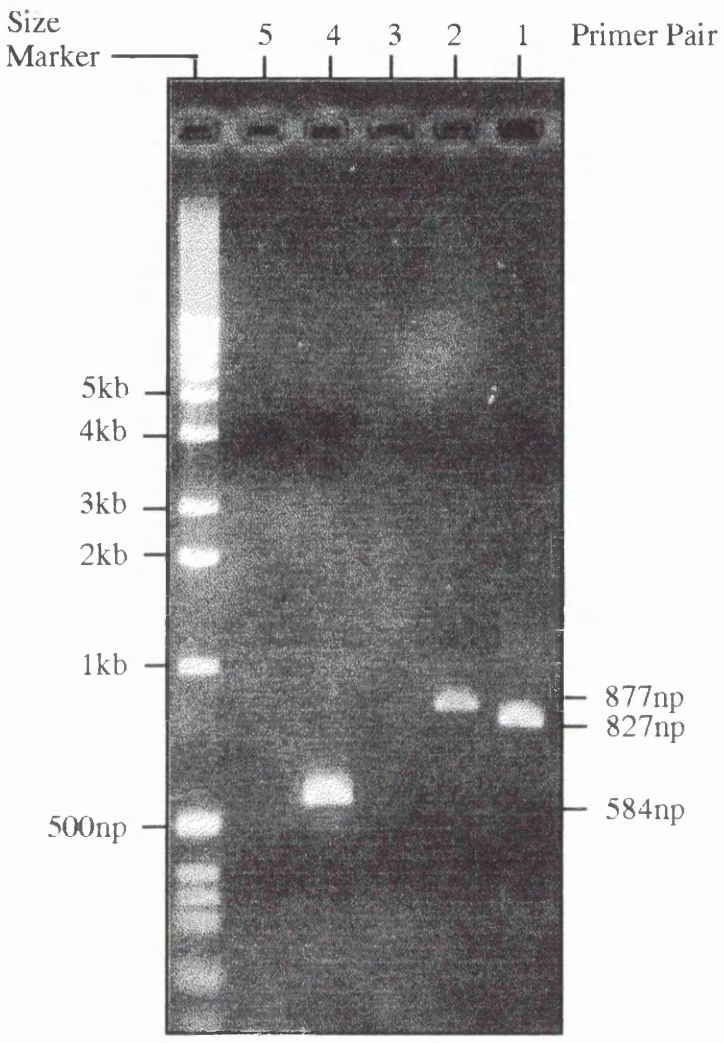


Figure 3:4 PCR amplificationof template DNA from patient number 1. Primer pairs 1-5 correspond to those shown in Table 3:3. The control PCR reaction (without DNA) is not shown - no amplification product was present.

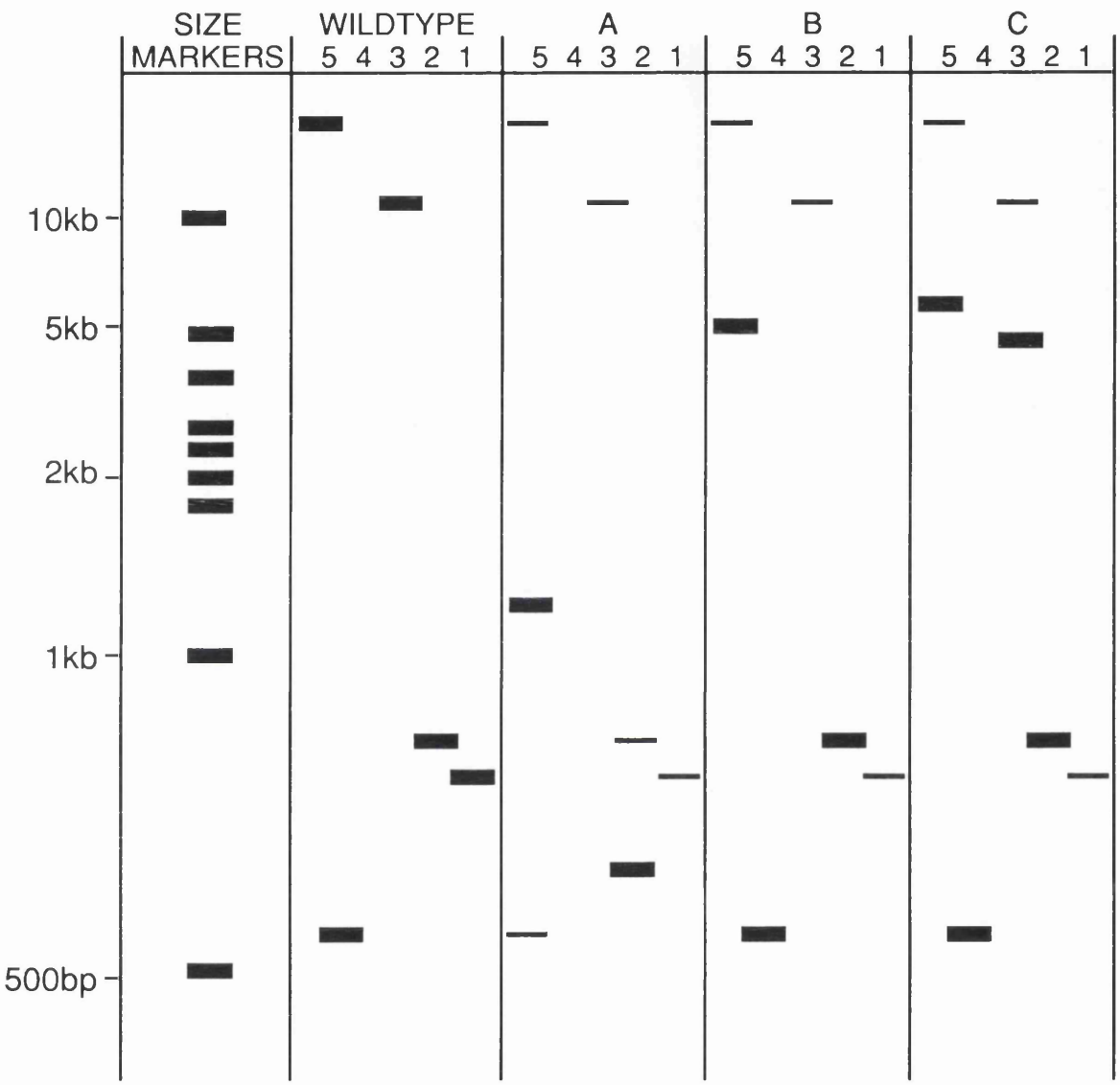


Figure 3:5 Diagramatic representation of the PCR products expected using primers given in Table 3:3, in the presence of three previosly described deletions, A, B and C, the breakpoints of which are given in Table 3:3.

Bands shown in bold would be those expected, others in samples A, B and C, reflect wild-type bands which would still be present.

duplication or deletion is present. Patients 2,16,33,36,49 and 112 (see Table 3:1) were investigated in this way and similar results obtained.

3:1:7 Discussion of results obtained in study of patients suffering from sensorineural hearing loss with no known aetiology

Clinical manifestations of mitochondrial disorders are extremely variable as described in Chapter 1. The auditory system has a high energy demand and requires efficient oxidative phosphorylation; it is among the first tissues to be affected by a decrease in oxidative phosphorylation (Elverland & Torbergsen, 1991). In accessibility of the sensory structures in the ear (mainly the cochlea) results in a poor understanding of the biochemical deficiencies which lead to deafness, either solely or in association with other symptoms. Sensorineural hearing loss is often said to be the initial symptom in mitochondrial disease, but often no details regarding the audiometric status of the patient is given. Deafness is a symptom described in a number of mitochondrial diseases: MELAS (Macmillan et al, 1993), Kearns/Sayre (Shoffner et al, 1989), MERRF (Shoffner et al, 1990), and in association with diabetes (Ballinger et al, 1992; van den Ouweland et al, 1992).

This study of patients suffering from sensorineural hearing loss with no known aetiology was initiated by Mr Andreas Nicolaides at the Victoria Infirmary, Glasgow and continued after his departure by Mr Guy Vernham. A number of mitochondrial RFLPs previously identified in association with various mitochondrial diseases were investigated.

The results of this analysis, given in Table 3:2, showed that one patient, number 31, had lost the *XbaI* site at 7440, an RFLP which had previously been found in association with LHON (Brown et al, 1992). The results of the detailed investigation of this patient are described in Chapter 4 of this thesis.

The remaining 32 patients had normal (wild type) sized restriction digestion products with all other enzymes used. There are obviously additional disease associated enzyme sites which could have been screened by RFLP analysis but time did not permit this.

It should be borne in mind that, in general, for a restriction site gain caused by a disease associated mutation only these particular point mutations would be detected in our study. A different point mutation within the same restriction site would go undetected. In view of the two different point mutations, at np 8993,

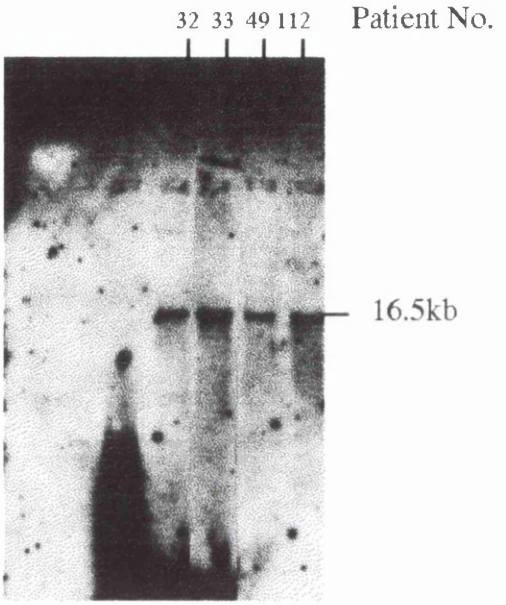


Figure 3:6 Southern blot of mitochondrial DNA from patients 32, 33, 49 and 112 cut with *Pvu*II and hybridised with a DIG-random labelled PCR product created using primers FR31/7178B and FR32/7840L.

which have now been found in NARP (Santorelli et al, 1994) this is an important point.

A number of patients were investigated for the presence of large scale rearrangements of their mitochondrial DNA. Figure 3:4 is typical of the results obtained, by Southern analysis, for all patients screened in this way. The single band of 16 kb is linearised mitochondrial DNA. Small rearrangements would not be detected using this technique. The limits of detection of this method are difficult to assess,. It obviously depends on the resolution of different sized DNA molecules during electrophoresis through agarose. Rearrangements altering the size of the mitochondrial genome by less than 1 kb would, by my estimation, be difficult to detect using this method. As discussed in Chapter 1 the presence of duplications has been underestimated in a number of previous investigations (Poulton et al, 1994). Restriction digestion using a number of different enzymes followed by Southern blotting and probing with labelled total mitochondrial DNA would reveal duplications and deletion monomers where present.

The entire mitochondrial genome can be amplified using optimised PCR conditions (Cheng et al, 1994). This will facilitate the use of PCR in the investigation of rearrangements of mitochondrial DNA. In the limited study carried out on 4 of the patients in Table 3:2, large scale deletions could be excluded; bearing in mind that the limit of the length of PCR product possible, using PCR conditions outlined in Chapter 2:6 is approximately 3 kb, small deletions would not be detected using the PCR primers given in Table 3:3. PCR conditions which enable amplification of the entire mitochondrial genome can easily be used to screen patients for rearrangements of their mitochondrial DNA, using a relatively small number of PCR primers.

Three of the 33 patients investigated (numbers 1, 2 and 31) had a maternal relative who was also deaf, consistent with a mitochondrial defect being associated with their development of deafness. Other explanations are possible, e.g. inheritance of an autosomal dominant deafness trait, exposure to similar environmental agents. With the advances in automated sequencing technology the entire mitochondrial genome of patients 1 and 2 could be sequenced relatively rapidly, to reveal any novel polymorphisms which may be further investigated for their association with sensorineural hearing loss.

In conclusion, no patient in this survey (apart from number 31) had any of the mitochondrial DNA mutations screened. Their non-syndromic deafness cannot, therefore, be associated with either the common MELAS or LHON associated mutations, based on the results presented here. It is possible, however, that

previously identified mutations in mitochondrial DNA are present in some of these patients. Further investigations should be undertaken to screen for many more mutations e.g. 8993 in NARP, identified using restriction enzymes *Ava*I and *Msp*I (Santorelli et al, 1994). Another possibility is to use SSCP (see Chapter 4) to screen all of the patients.

In view of the large number of neurological symptoms found in mitochondrial disease, and the fact that deafness is a common first symptom, it would be surprising if some of the patients in this study did not have other disease-associated mutations.

3:2 INVESTIGATION OF PATIENTS SUFFERING FROM AMINOGLYCOSIDE ANTIBIOTIC-INDUCED SENSORINEURAL HEARING LOSS

In 1991 Hu et al reported an epidemiological study of 36 pedigrees in which there were patients with AAID and found that in 22 the trait was maternally transmitted. They concluded that in these 22 pedigrees there may be a mutation of mitochondrial DNA which is associated with the development of deafness. This study prompted us to look at DNA from AAID patients for the presence of some previously identified mitochondrial DNA mutations.

3:2:1 Medical history of Chinese patients

Eighteen patients suffering from sensorineural hearing loss, which developed after treatment with aminoglycoside antibiotics, were selected by Dr Wei-Qin Qiu of Shanghai. DNA was prepared from blood samples in Shanghai using a protocol provided by our laboratory (Section 2:4) and the DNA transported to Glasgow as a dry precipitate. Table 3:4 gives details of the patients as supplied by Dr Qiu: no information regarding which aminoglycoside, the dose or the duration of the treatment, was provided. Although the intention was to study patients belonging to pedigrees in which the AAID was maternally inherited, the DNA which Dr Qiu brought was from sporadic cases. In addition we are unaware of the degree of deafness suffered by these patients or whether the hearing loss was bilateral.

3:2:2 Medical history of Scottish patients

Three patients with sensorineural hearing loss who had been treated with aminoglycosides whilst in babycare at the Royal Infirmary, Glasgow were selected by Professor George Browning. Gentamycin was the antibiotic used in

Patient	Age at Examination	Age at Treatment	*Xba*I site at np 7440	Genotype at np 1555
CH 1	8 y	10m	wt	wt
CH 2	8 y	1m	wt	wt
CH 3	7 y	6.5m	wt	wt
CH 4	9 y	3y	wt	wt
CH 5	9 y	1m	wt	wt
CH 6	8 y	?	wt	wt
CH 7	9 y	2y	wt	wt
CH 10	10 y	1y	wt	wt
CH 11	12 y	1y	wt	wt
CH 12	11 y	1y8m	wt	wt
*CH 13	16 y	3y	wt	A->G
*CH 14	16 y	3y	wt	A->G
CH 15	7 y	1y	wt	wt
CH 16	6 y	8m	wt	wt
CH 17	6 y	1y2m	wt	wt
CH 18	6 y	1y	wt	wt
CH 19	7 y	2m	wt	wt
CH 20	7 y	4y	wt	wt

Table 3:4 Data from aminoglycoside-treated Chinese patients. The *Xba*I site was screened by restriction digestion of the 662 np fragment created by PCR using FR31/7178B as forward primer and FR32/78404 as reverse primer. The genotype at np 1555 was determined by direct sequencing of PCR products.

y-years; m-months; wt-wild type; *CH13 and CH14 are identical twins.

each case, doses ranging from 7 mg/day to 15 mg/day for a duration of 1 day to 4 days: Table 3:5 shows the age of examination. Each patient completed the questionnaire; there was no history of diabetes, epilepsy, muscle weakness or serious eye problems. Patient 115 reported that she suffers from episodes of migraine, as does her two brothers and her mother.

DNA was prepared from blood of patients number 113 and 114 and from buccal cells in a mouthwash sample from patient 115 (Section 2:4).

3:2:3 RFLP analysis of mitochondrial DNA from patients suffering from aminoglycoside antibiotic-induced deafness (AAID)

The *Xba*I site loss at np 7440 of mitochondrial DNA previously reported in LHON patients (Brown et al, 1992) and found in one patient (number 31) in our study of non-syndromic deaf patients, was investigated in both Chinese and Scottish patients in a similar manner to that given in Section 3:1:3. As Tables 3:4 and 3:5 show, in no AAID patient had this *Xba*I site been lost.

During his time working in Glasgow Dr Wei-Qin Qiu screened a small number of the Chinese AAID patients for the mutation site at np 3243, detected by *Apa*I site gain and that at 3460 detected by *Aha*II site loss. No change from wild type was found in any patient tested, but Dr Qiu left no written data in Glasgow when he returned to China.

3:2:4 Sequence analysis of Chinese patients in the region around np 7440

In view of the sequence change causing loss of the *Xba*I site, found in patient 31 (see Chapter 4), the region of the mitochondrial genome around the *Xba*I site at np 7440 was sequenced in the Chinese AAID patients. A PCR product of 662 np was amplified using primers FR31/7178B and FR32/7840L and the sense strand sequenced using primer FR49/7324L. No nucleotide change in this *Xba*I site was found in any of the patients, but as Figure 3:7 shows a T->C change at np 7389 was seen in patient CH18. This change was confirmed by sequencing the other strand. This mutation changes a Tyrosine to a Histidine and the nucleotide sequence change creates an *Nla*III restriction site. The remaining Chinese patients were all wild-type at this site.

3:2:5 Evaluation of the genotype at np 1555 in AAID patients

As a result of a report by Prezant et al (1993) of an A->G homoplasmic change at np 1555 in the mitochondrial DNA of patients with maternally transmitted AAID,

Patient	Age at Examination	Age at Treatment	*Xba*I site at np 7440	Genotype at np 1555
113	14 y	1d	wt	wt
114	13 y	1d	wt	wt
115	14 y	1-4d	wt	wt

Table 3:5 Aminoglycoside treated Scottish patients.
y-years; d-days.

Treatment was with Gentamycin at a dosage of 7-15 mg/day/1-4 days after birth.

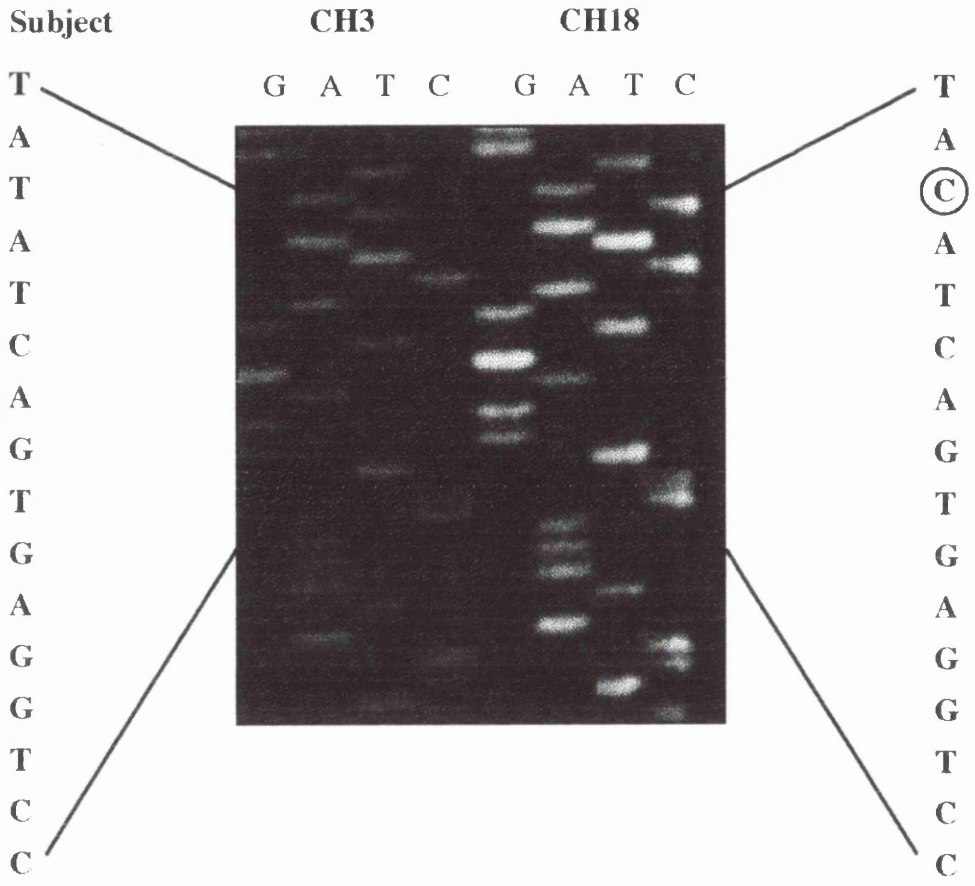


Figure 3:7 Direct DNA sequencing of the region around np 7440 in patient CH3 and Ch18. A PCR product of 662 np was amplified using forward primer FR31/7178B and reverse primer FR32/7840L. The sense strand is shown with the T->C at np 7389 in patient CH18 indicated by a circle.

we decided to screen the AAID patients available to us for this mutation. A 682 np amplification product from the 12S rRNA gene was prepared using FR12/1444B as forward primer and FR13/2125L as reverse primer. After purification the biotinylated strand of the amplified product was sequenced using the primer FR69/1705L (Section 2:10). Patients CH13 and CH14, who were identical twins, were found to have the same homoplasmic A->G change at np 1555 reported by Prezant et al (1993). No other AAID patient tested had this mutation, and no other mutation was found in this region of the 12S rRNA gene in any AAID patient.

Figure 3:8 shows the homoplasmic mutation on the antisense strand of patient CH14 alongside the normal sequence of patient CH5.

3:2:6 Discussion of results obtained in the survey of AAID patients of Chinese and Scottish origin

3:2:6:1 Association of aminoglycoside antibiotics and development of deafness

Aminoglycoside antibiotics, in particular Streptomycin, but also Kanamycin, Gentamycin, Tobramycin and Neomycin, are known to be ototoxic and nephrotoxic, streptomycin being less nephrotoxic than the other aminoglycosides (Appel and Neu, 1977). The loss of hearing which can result from such treatment is related to dosage, age of subject, renal function and inherited predisposition. Streptomycin sulphate was widely used to treat pulmonary tuberculosis in the 1950s and 60s. In some instances it was used to treat much less serious infections; in Japan bacterial infections following the common cold were treated with streptomycin (Higashi, 1989). When the toxic effects of aminoglycosides became evident its use was restricted in many countries.

Some patients seem to have an inherited predisposition to develop deafness after a single short course of treatment. The pattern of inheritance in many cases appeared to be compatible with autosomal dominance. Viljoen et al (1983) studied a large kindred in which there were eight persons, over four generations, with bilateral sensorineural hearing loss attributed to aminoglycoside treatment. The pattern of inheritance was claimed to be autosomal dominant but re-examination of the pedigree shows it to be compatible with maternal transmission. Higashi (1989) compiled 28 pedigrees in which deafness following aminoglycoside treatment was inherited only from females or through the female line. In many of these families the pattern of inheritance had previously been categorised as autosomal dominant.

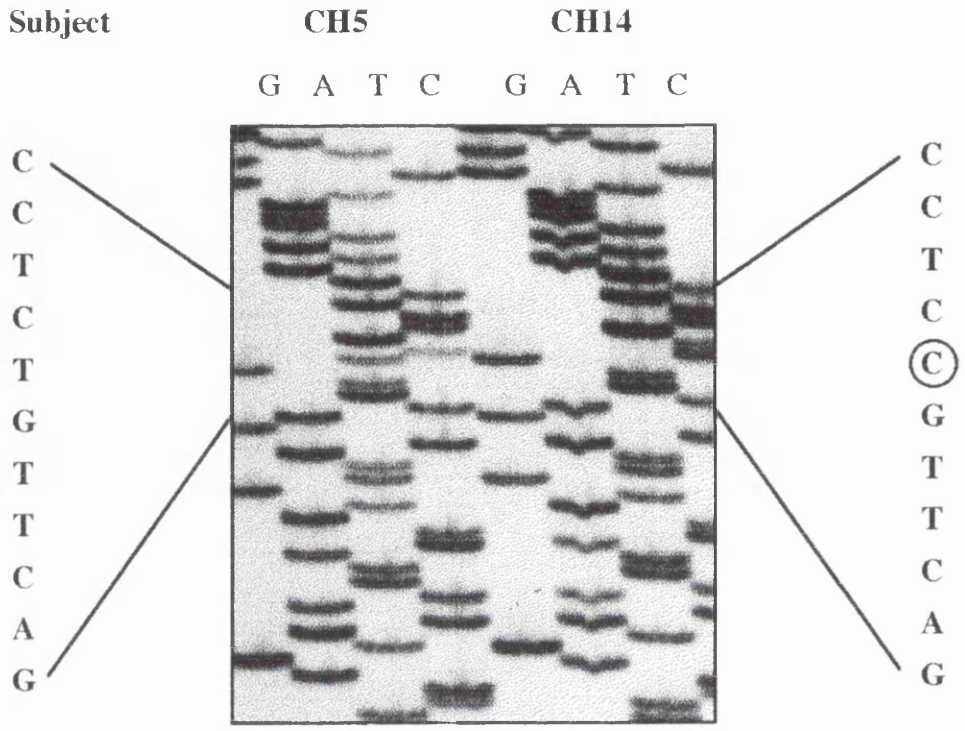


Figure 3:8 Direct DNA sequencing of a PCR product derived from the 12S rRNA gene from patient CH14 (mutant) and CH5 (wild-type). Sequence shown is of the antisense strand. The T-> C mutation at np 1555 is indicated by a circle.

Aminoglycosides are still widely used in China to treat a variety of infections: Hu et al (1991) made a study of 36 pedigrees with AAID. In 22 of these pedigrees, transmission of the predisposition to become deaf after antibiotic treatment was exclusively through females. In the remaining 14 pedigrees transmission could not be ascertained. This report prompted us to contact the Chinese group in Shanghai and arrange a collaborative study of some of their families. Blood was obtained and DNA prepared in Shanghai (under the direction of Dr Wei-Qin Qiu who subsequently brought the samples to Glasgow) from a collection of 18 patients with sporadic AAID.

3:2:6:2 Sequence changes identified in AAID patients

The presence of the *Xba*I site at np 7440 was screened in the 18 Chinese patients and 3 Scottish patients. As Tables 3:4 and 3:5 show no change from normal was found in any of the patients.

Sequencing of all 18 Chinese AAID patients in the region encompassing the 3'-end of the COI gene and the 3' end of the $tRNA_{ser}$ gene, revealed that patient CH18 has a T->C mutation at np 7389. This is in the COI gene and causes an amino acid substitution, but as Figure 3:9 shows there is poor conservation of the amino acid at this position. The nucleotide change creates an *Nla*II restriction enzyme site and this could be used to screen normal controls, non-syndromic deaf patients, sporadic and maternally inherited AAID patients to establish whether this mutation is simply an uncommon polymorphism. None of the members of pedigree or normal controls, described in Chapter 4, who had this region of their mitochondrial DNA sequenced had the T->C change at np 7389.

In July 1993 Prezant et al published a report of a significant nucleotide change in the mitochondrial DNA of three unrelated patients with familial AAID. This mutation is an A->G change at np 1555 in the 12S rRNA gene; it had not been identified in more than 270 controls. The mutation was also identified in a large Arab-Israeli kindred with maternal transmission of deafness but no association with the use of antibiotics.

As a result of this report the three Scottish and eighteen Chinese samples were screened by means of PCR and direct sequencing, for the mutation at np 1555. As Table 3:4 shows, the A->G mutation at np 1555 was present in 2/18 Chinese patients; these two patients are identical twins. Figure 3:8 shows the homoplasmic A->G change at np 1555 in patient CH14 compared to the normal sequence in patient CH5. All the Chinese patients, with the exception of CH13 and CH14, had no family history of aminoglycoside induced deafness, i.e. they

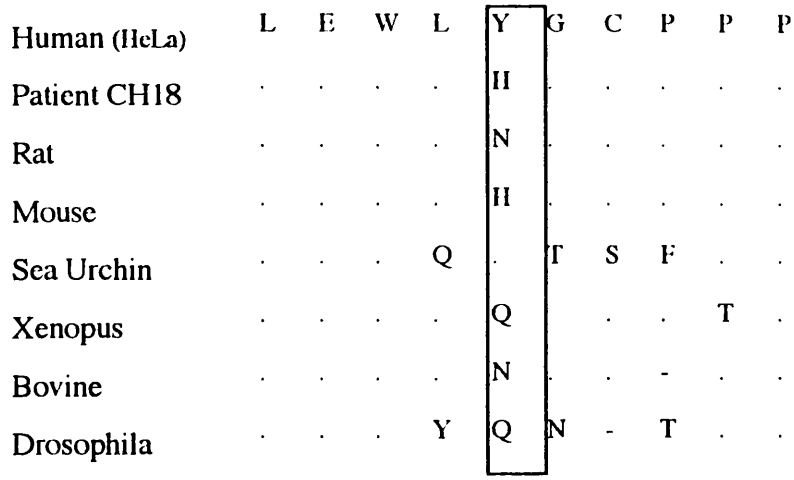


Figure 3:9 Amino acid sequence of the region of COI from amino acid 492 to 502. Human sequence of HeLa cells is aligned with the counterpart in Patient CH18, rat, mouse, sea urchin, *Xenopus*, cow and *Drosophila*. The dots represent identity, the dashes that no amino acid is assigned to that position. The boxed area shows the lack of conservation of amino acid 496.

were sporadic cases. The three Scottish AAID patients were also sporadic and were found by direct sequencing to have normal sequence at np 1555.

The A->G at np 1555 has now been found in 5 pedigrees with AAID (Hutchin et al, 1993); it has never ocurred in 414 hearing people studied. This mutation results in the loss of a *Bsm*AI restriction site providing a potentially rapid means of screening a large number of individuals.

3:2:6:3 Mechanism of action of streptomycin

The precise mechanism and site of action of aminoglycosides, which lead to the development of deafness, is unclear. Damage is thought to be to the hair cells in the Organ of Corti (Ballantyne, 1970) which lies within the cochlea (see Figure 3:10). Streptomycin is also known to damage the nerve fibre and ganglion cells.

The molecular mode of action of aminoglycosides (as antimicrobial agents) has been elucidated by studies using *E. coli* and streptomycin, clinically the most commonly used aminoglycoside. Streptomycin has been shown to interfere with bacterial protein synthesis by binding to the small (30S) subunit of the prokaryotic ribosome (Cox et al, 1964).

The 30S subunit of *E coli* ribosomes is a ribonucleoprotein complex consisting of 21 proteins and 16S rRNA. The protein subunits designated S4, S5 and S12 have been mapped by immuno-electron microscopy to a site within the ribosome where codon-anticodon interaction takes place. This maps to the region of 16S rRNA from nucleotide 1400-1500 (Stern et al, 1988). If 30S ribosomal subunits are reconstituted, *in vitro*, without S12 protein they no longer bind streptomycin (Ozaki et al, 1969). When no drugs are present such S12-deficient ribosomes are less prone to mis-reading than normal ribosomes; therefore it appears that protein S12 is in part responsible for inherent infidelity of translation and that this is enhanced by aminoglycosides.

Mutations in the ribosomal protein S12 have been shown to confer Streptomycin resistance or dependance in *E. coli* (Allen & Noller, 1989). Specific residues in the 530 stem and loop of 16S rRNA are protected by S12 in chemical protection assays (see Figure 3:10) indicating that S12 is bound in this region (Finken et al, 1993) and probably exerts its influence by perturbing the 3D structure of 16S rRNA.

Assays in which 16S rRNA has been shown to be protected from RNA-specific chemical reagents, such as dimethyl sulphate, by streptomycin indicate that

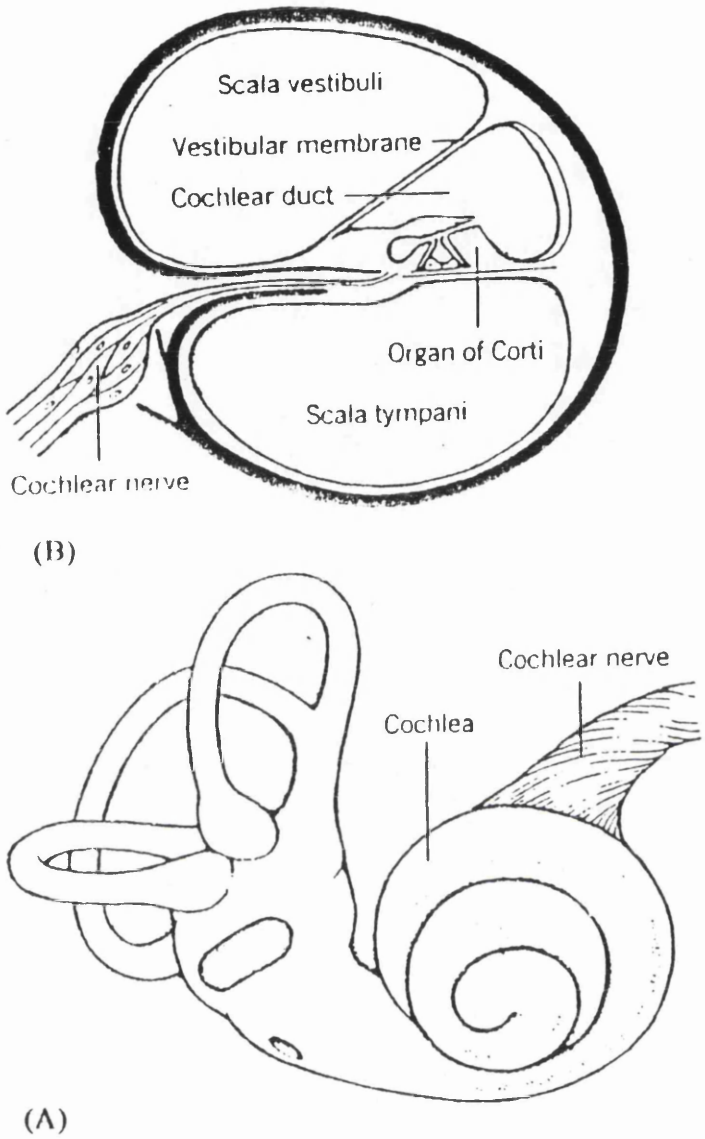


Figure 3:10 Human cochlea (A), showing the cochlear nerve, and cross-section (B) showing the position of the Organ of Corti within the cochlea. Reproduced from Churchill's Medical Dictionary.

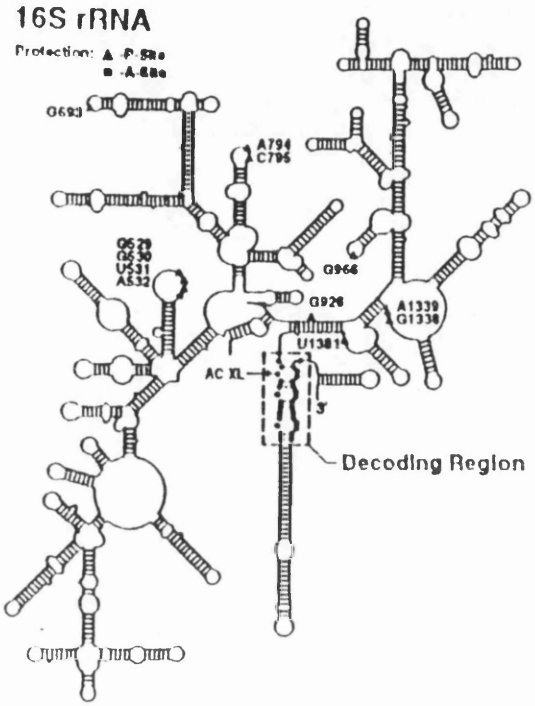


Figure 3:11 Schematic secondary structure of 16S rRNA of *E. coli* taken from Purohit and Stern (1994).

streptomycin protects a group of residues in the 915 region of *E. coli* 16S rRNA (O'Connor et al, 1991). Mutations at nucleotide 1491 in the 16S rRNA of *E. coli* abolish Watson & Crick base pairing to the C-nucleotide at 1409 (De Stasio et al, 1989) and confer resistance to streptomycin. Since mitochondria are thought to be derived from a eubacterial endosymbiont, the mitochondrial ribosomes are more akin to prokaryotic ribosomes than to cytosolic ribosomes. It is likely, therefore, that mitochondrial ribosomes will be susceptible to the action of antibiotics which act on prokaryotic ribosomes. Figure 3:12 shows the 16S rRNA of *E. coli* in the region 1392-1534 in comparison with the 3' end of human mitochondrial small subunit 12S rRNA. The position np 1555 in 12S rRNA gene at which the mutation in some AAID patients has been mapped is structurally equivalent to position 1491 in *E. coli* 16S rRNA. The A->G change at nucleotide 1555 will permit Watson - Crick base pairing to the C at nucleotide 1494 of 12S rRNA creating a structure in the mitochondrial 12S rRNA very similar to the region in *E. coli* 16S rRNA outlined by O'Connor et al (1991) as a region with which streptomycin interacts. Thus the change at this position facilitates the binding of streptomycin to the small subunit of the mitochondrial ribosome where it could play a role in impairing fidelity of translation analogous to its role in bacteria.

It has been confirmed in this study that some sporaidc AAID patients have an A->G change at np 1555, while others who apparently suffer the same symptoms, do not. It is possible that there are other mutation sites within the 12S rRNA of the remaining Chinese and Scottish patients which confer aminoglycoside sensitivity by a different, but related mechanism. Identification of other mutations could only be achieved by direct sequencing of the entire gene of each patient. An attractive possibility is that other mutations exist which play a role in aminoglycoside binding, or in the binding of one of the ribosomal proteins as with protein S12 in *E. coli.*

3:2:6:4 Final conclusions

As indicated by Ballantyne (1970) deafness is thought to result from the death of hair cells within the Organ of Corti. Exactly how mistranslation of mitochondrial proteins brings this about is still unknown. Hutchin et al (1993) suggest that hair cell death is due to excitotoxicity.

Albin and Greenamyre (1992) suggested that neuronal death could be intiated by impairment of cellular metabolism which, in turn, leads to failure of the Na/K ATPase which maintains normal membrane potential. When the membrane becomes depolarised the Mg 2+ion blockade of receptors for excitotoxic amino

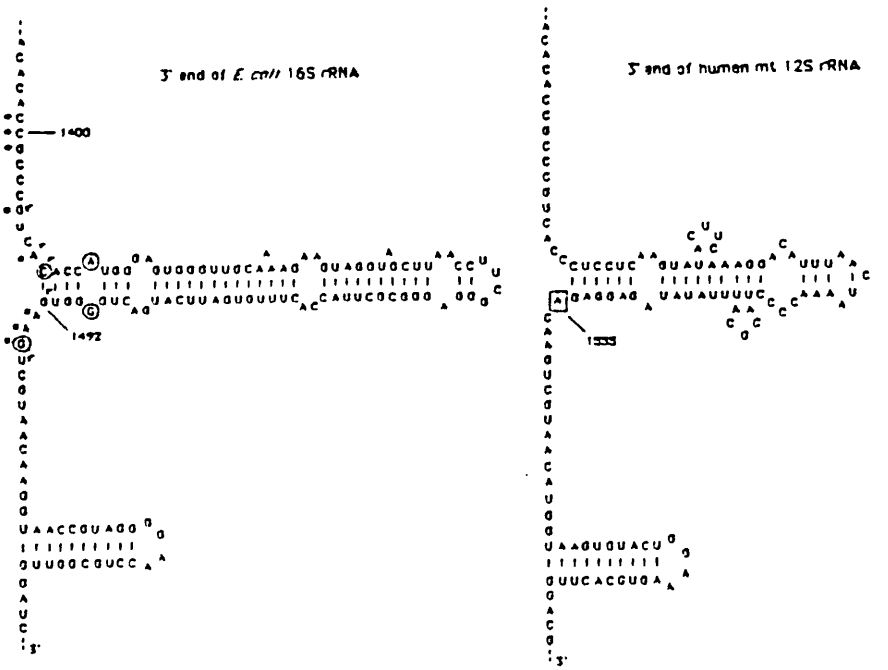


Figure 3:12 The 3' end of human mitochondrial 12S rRNA in comparison with the 3' end of *E. coli* 16S rRNA. The position of the A->G at np 1555 in human mitochondrial 12S rRNA is indicated. Taken from Hutchin et al (1993).

acid (EAA), such as Glutamate, is reduced, allowing receptor activation and subsequent excitotoxic effects.

It remains unclear why it is only the neurons involved in the auditory system that are affected by aminoglycosides. As with most mitochondrial diseases, the tissue specific pathology accompanying the np 1555 mutation cannot be explained without invoking a role for some external factor. The presence of the np 1555 mutation in the large Arab-Israeli kindred, with no involvement of aminoglycosides, leads to the conclusion that this mutation in association with either an external factor, e.g. streptomycin, a set of other mt DNA sequence polymorphisms, or a nuclear gene product results in the phenotype of sensorineural deafness.

There is obviously much scope for further investigation of the mechanisms involved in the development of disease in AAID.

CHAPTER 4 STUDY OF SENSORINEURAL HEARING LOSS WITHIN A LARGE MATERNAL PEDIGREE

4:1 IDENTIFICATION OF THE FAMILY

During the survey carried out at the Victoria Infirmary, Glasgow, of patients suffering from sensorineural hearing loss (outlined in Chapter 3) a female aged 11 (patient number 31 in Table 3:1) with moderate bilateral deafness since age 3 was identified, whose mother was also known to have a hearing problem. The mother had been diagnosed at age 18 and was found when re-examined audiometrically, as part of this study, to have a mild bilateral hearing loss. Neither the mother nor child had ever had any episode of migraine, epilepsy, muscle weakness; neither is diabetic.

Via the questionnaire the mother revealed that one of her three sisters, a number of nieces and a nephew had hearing problems. These members of the immediate family were contacted and attended the Victoria Infirmary for audiometric testing and donation of blood samples. Completed questionnaires indicated that there are branches of the family elsewhere, in Australia and in Southern Africa including Zimbabwe, some of whom were thought to suffer from deafness.

4:2 MEDICAL HISTORY OF THE PEDIGREE

The maternal pedigree of this family, as far as it is now known, is shown in Figure 4:1. Additional information was supplied by II-8 regarding the deceased parents of generation I: the mother was born in Poole, Dorset circa 1888 and the father in Lochgoilhead, Argyll circa 1870. There is no knowledge of their hearing status. Of generation I only I-6 remains alive, presently residing in Australia. This woman is known to have mild hearing loss, but this is probably presbycusis as she is now over 70 years old. Individual II-2 is in Zimbabwe where he was contacted by telephone and confirmed that none of his two children (III-4 and III-5), his brother (II-1), nor his brother's children (III-1, III-2 and III-3) has any hearing problem. To his knowledge his now deceased father (I-1) also had no history of deafness. Subject II-3 has no hearing loss but is reported to have an olfactory problem: she has no sense of smell. Of her three sons only one (III-6) has a history of deafness: he was diagnosed as suffering from bilateral sensorineural hearing loss at age 9 and has had progressive loss since that age - he is now 34. His mother recalls him having suffered from German measles at approximately the same age as his deafness was diagnosed, but no connection was ever suggested by clinicians.

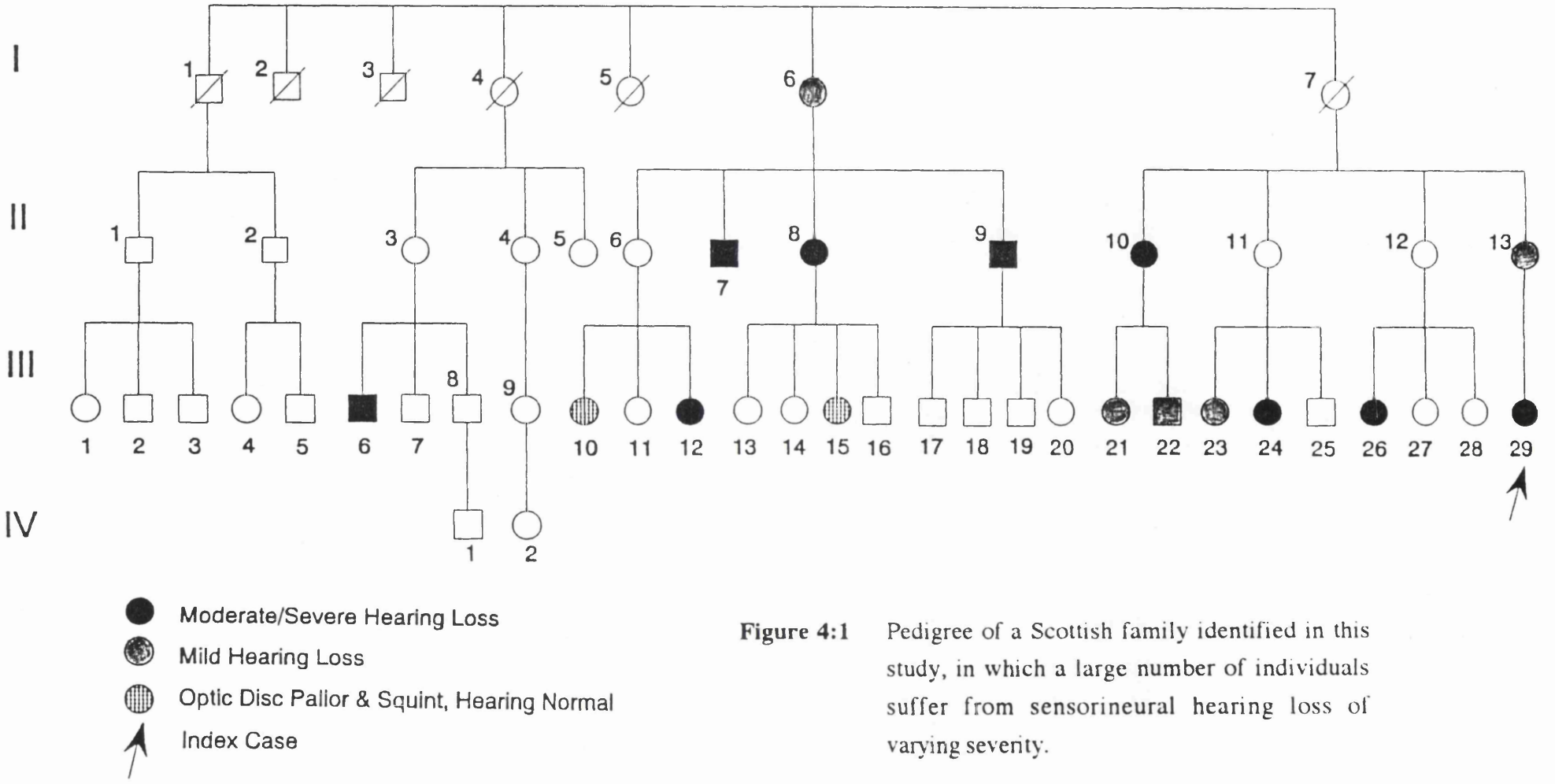


Figure 4:1 Pedigree of a Scottish family identified in this study, in which a large number of individuals suffer from sensorineural hearing loss of varying severity.

Neither of the sisters of subject II-3, i.e. II-4 and II-5 are reported to have hearing problems. II-4 is now resident in Canada where she has been contacted. Neither she nor her daughter (III-9) would agree to be audiometrically tested. The remaining sister (II-5) lives in Scotland but refused to respond to any enquiry, from her sister, by letter from us or through her own General Practitioner.

A great deal of information has been provided about the branch of the family resident in Australia (I-6, II-6, II-8, II-9 and their children) from subject II-8. This family member has had a gradual progression of hearing impairment since her mid twenties, but had had no auditory test since 1977 when she was examined at the Ear, Nose and Throat Hospital, Glasgow. On that occasion she was told that there was auditory nerve involvement. Although there is no evidence of hearing problems in the four children of II-8 (III-13, III-14, III-15 and III-16), III-15 has visual impairment. Dr G Wards of Penrith, NSW has reported that she has optic-disc pallor in her left eye, micro left divergent squint with added exophoria for near and distance and her left visual acuity (LVA), with glasses, is 6/9 - the normal value is 6/6. The sister of II-8, II-6 has no hearing problem but one of her daughters (III-12) has had hearing impairment since infancy: this child wears hearing aids in both ears. Another of the three daughters (III-10) has a problem with her vision very similar to that of III-15, with optic disc pallor in both eyes, left squint and LVA with glasses, of 6/18 but no hearing loss (information provided by Dr G Wards).

Both brothers of II-8 suffer from moderate hearing loss: II-9 has been deaf since infancy when he contracted measles at 5 months old. None of his four children have hearing problems; it is worthy of comment that the maternal grandparents of these four children are both profoundly deaf. This illustrates the potential for difficulties in studies of inherited deafness where there is intermarriage of two families with entirely different aetiologies of deafness.

The second brother of II-8, subject II-7, resides in Leicester, England. He has not recently had his hearing tested, but has informed us that he has worn a hearing aid since age 8 and since 1986 has had one for both ears. His mother, I-6, recalls that he suffered from measles as a child, but cannot clearly remember the details.

The remainder of the family live in or around Glasgow and have all been examined by Mr G Vernham at the Victoria Infirmary. Subject II-10 suffers from moderate bilateral hearing loss but had not admitted to having a hearing problem until recently; consequently age of onset is unknown. Her son and daughter (III-22 and III-21) both have mild bilateral deafness which was diagnosed during their primary education. Two of the three sisters of II-10 (II-11 and II-12) show no

evidence of deafness, but have offspring who are moderately deaf. Of the children of II-11, III-24 has moderate bilateral loss which was diagnosed at the age of 6 and is progressive. Her sister, III-23 has a mild bilateral hearing loss which is also thought to be progressive.

One of the daughters of II-12 has a moderate loss of hearing which was detected at age 10; she wears a hearing aid and attends the Victoria Infirmary for monitoring of the progression of her deafness. Individuals III-29 and II-13 are the child and her mother whose replies to the questionnaire brought our attention to the family.

No member of the pedigree has been reported to suffer from epilepsy, muscle weakness or diabetes. A small number report migraine episodes but no more than in the general population.

The deafness in this family appears to be maternally inherited (Figure 4:1) leading to speculation that a mitochondrial defect could be involved in the development of the disease phenotype. In order to investigate this possibility a more detailed audiometric study of the family was initiated prior to investigation of their mitochondrial DNA.

4:3 PURE TONE AUDIOMETRY OF MEMBERS OF THE PEDIGREE

Pure tone audiometry was carried out in Glasgow at the Victoria Infirmary by the staff of the Ear, Nose and Throat Department under the direction of Mr Guy Venham; in Australia at Wollongong Hearing Centre under the direction of Dr Patrick Rundle. The results on the twenty three members of the pedigree tested are summarised in Table 4:1. On advice from Mr Vernham mild hearing loss was defined as a bilateral average of 20-35 dB, moderate loss as 35-85 dB and severe loss as greater than 85 dB. Only one individual (II-9) had severe bilateral hearing loss; 8 of the 23 showed moderate loss. Including the 5 who had mild bilateral deafness, 14 of the 23 maternally related members of the pedigree tested had some degree of hearing impairment.

In addition to the 23 results given in Table 4:1 audiometric testing of III-17, III-18, III-19 and III-20 was carried out. These children are maternally unrelated to the rest of the family; the results showed no evidence of sensorineural hearing loss. A typical audiometric trace from one of the moderately deaf individuals is given in Figure 4:2, showing a marked high frequency impairment.

Patient	Age at examination	Pure Tone Average[a] Right	Left	Hearing[b]
I-6	73	21	33	Mild loss
II-3	56	16	14	Normal
II-7	28	59	55	Moderate loss
II-8	47	46	39	Moderate loss
II-9	44	103	103	Severe loss
II-10	45	34	37	Moderate loss
II-11	43	8	13	Normal
II-12	41	17	14	Normal
II-13	34	18	27	Mild loss
III-6	32	48	50	Moderate loss
III-12	5	33	40	Moderate loss
III-13	25	11	12	Normal
III-14	22	13	14	Normal
III-15	7	16	12	Normal
III-16	12	6	8	Normal
III-21	21	28	21	Mild loss
III-22	17	19	21	Mild loss
III-23	22	21	25	Mild loss
III-24	19	42	28	Moderate loss
III-25	13	12	13	Normal
III-26	21	62	64	Moderate loss
III-27	18	13	13	Normal
III-28	11	4	3	Normal
III-29	11	39	58	Moderate loss

a Averages at 0.25, 0.5, 1, 2, 4 & 8 for all subjects plus data at 1.5, 3 & 6 KHz for a minonity.

b Mild hearing loss defined arbitrarily as a bilateral average of 20-35 dB, moderate loss as 35-85 dB, severe loss as > 85 dB.

Table 4:1 Audiometric data for the index case, III-29, and her maternally related relatives, from measurements taken by staff of the Audiometry Department, Victoria Infirmary, Glasgow.

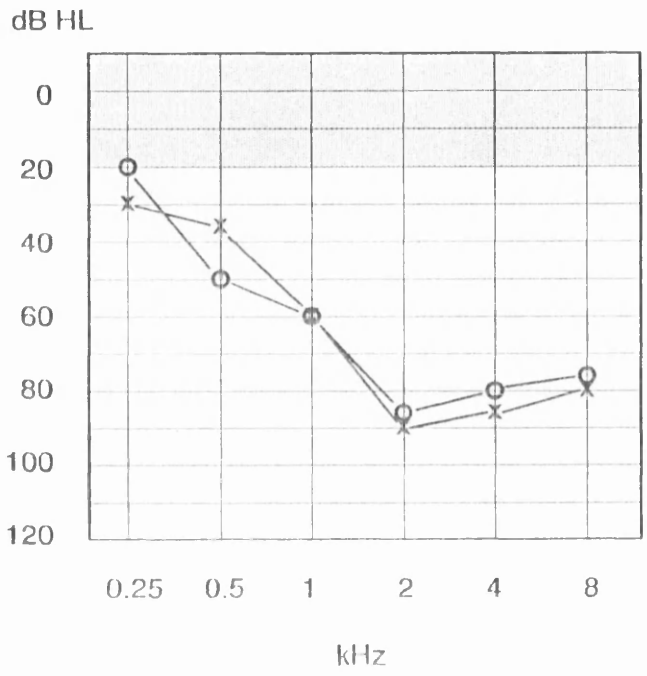


Figure 4:2 Audiometric trace. Pure tone audiometry of a typical, moderately deaf member of the pedigree (patient III-26). Hearing loss in decibels (dB) is shown for both right ear (o) and left ear (x).

The age of onset of hearing impairment in the family varied between 5 months and 18 years, with many patients having progressive loss. Figure 4:3 illustrates, however, that there is no obvious correlation between the severity of hearing loss and age at examination: there is also no obvious difference in the degree of hearing loss between the sexes.

4:4 COMPLETE NUCLEOTIDE SEQUENCE OF THE MITOCHONDRIAL DNA OF THE INDEX CASE

The apparent maternal inheritance of deafness within this family would be consistent with a mutation in the mitochondrial DNA having a role in the development of symptoms. Sequencing of the entire mitochondrial genome of one member of the pedigree was undertaken to identify any novel sequence polymorphism(s) which could be implicated in the disease. DNA was extracted from a blood sample obtained from the index case and, by means of PCR and direct sequencing of amplified products the entire mitochondrial DNA sequence established (see Chapter 2).

All deviations from the Cambridge sequence (Anderson et al 1981) were confirmed by sequencing on both strands: the polymorphisms found are listed in Table 4:2, all were homoplasmic, or nearly so, at least within the limits of detection of the method (estimated at less than 5%). Of the thirteen differences found, nine have previously been reported as probable mistakes in the Cambridge sequence. The remaining four deviations have not, to date, been reported elsewhere.

Two of these four lie with the D-loop:T->C at np 72 and T->C at np 195; neither position has been assigned a function. In Figure 4:4 the position of these 2 polymorphisms in relation to the major features of the D-loop is illustrated. Figure 4:5A shows 10 np upstream and 10 np downstream of the polymorphism at np 72, whilst Figure 4:5B shows the region 10 np upstream and 10 np downstream of the polymorphism at np 195; the proximity of this polymorphism to the origin of H-strand replication is obvious.

The A->G change at np 7445 is at a site which is assigned a function on both strands of human mitochondrial DNA. Figure 4:6 shows that on the major coding (H) strand this nucleotide is in the stop codon of the gene for cytochrome oxidase subunit I (COI), giving the silent AGA->AGG change. On the other strand (L) the np 7445 change alters the extreme 3' encoded nucleotide of tRNAser(UNC). This position is also part of an *Xba*I restriction enzyme site: *Xba*I cuts at 7440 the site being TCTAGA; this provides a simple method of screening for the

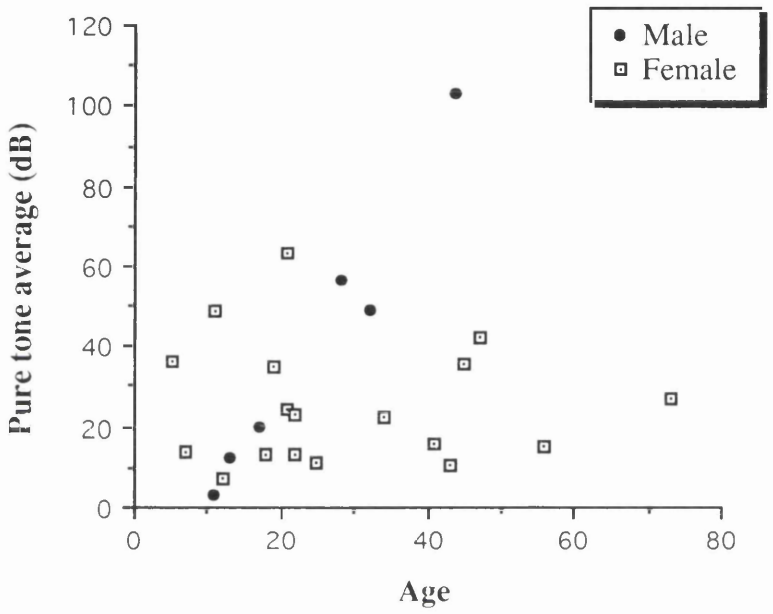


Figure 4:3 Plot of Audiometry versus Age at Examination.
Graph of pure tone average results against age at examination.
Showing no correlation.

Gene	Coordinate (np)	Change on H-strand	Amino acid substitution	Previously Reported?
D-loop	72	T->C	-	No
	195	T->C	-	No
12S rRNA	750	A->G	-	Yes Prezamt et al (1993)
	1438	A->G	-	Yes Marzuki et al (1992)
ND1	3423	G->T	Silent	Yes Howell et al (1992)
ND2	4985	G->A	Silent	Yes Howell et al (1992)
COI/tRNA-ser (UCN)	7445	A->G	Silent (COI)	No
ND4	11335	T->C	Silent	Yes Howell et al (1992)
ND6	14199	G->T	Pro->Thr	Yes Howell et al (1992)
	14365	G->C	Silent	Yes Howell et al (1992)
	14368	G->C	Phe->Leu	Yes Howell et al (1992)
cytochrome b	14766	T->C	Ile->Thr	Yes Lertrit et al (1992)
	15110	G->A	Ala->Thr	No

Table 4:2 Deviations from the Cambridge sequence of mitochondrial DNA from the index case (III-29).

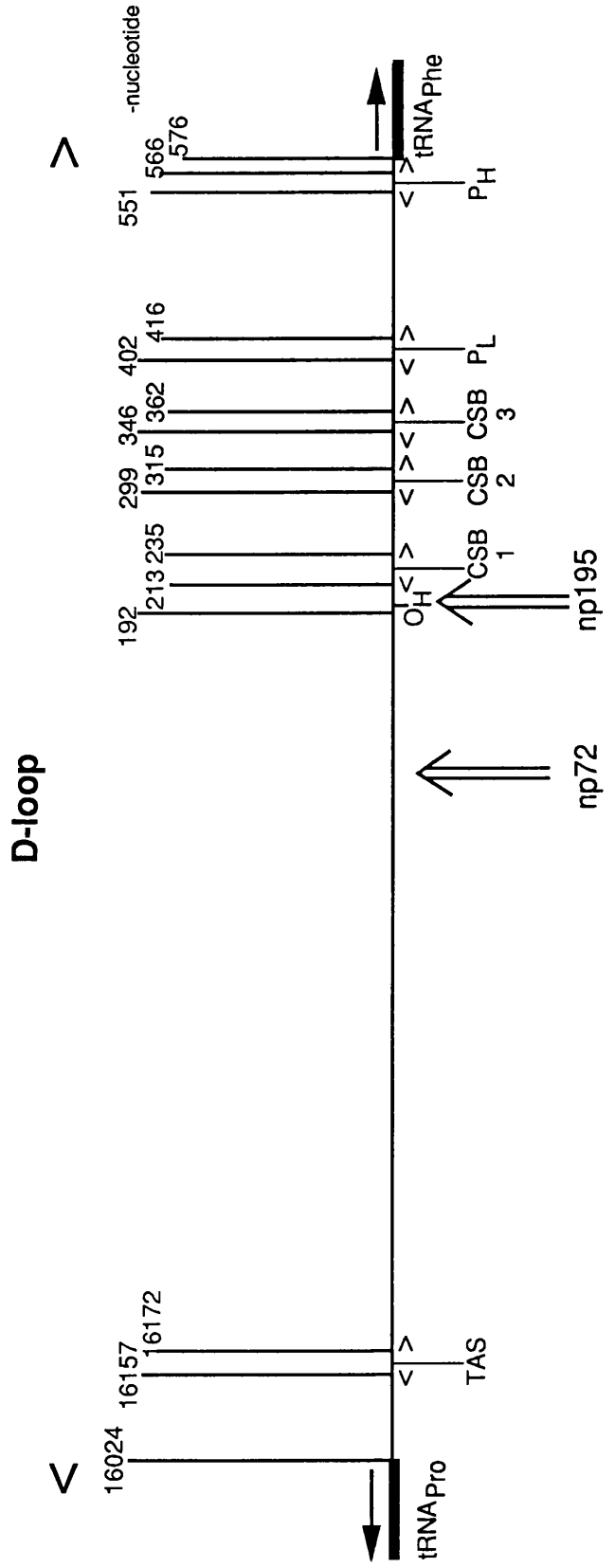


Figure 4:4 Illustration of the D-loop of human mitochondrial DNA showing the position of the deviations from the Cambridge sequence at np72 and np195 in relation to the major features of the D-loop: origin of light and heavy strand transcription (P_L and P_H) conserved sequencing boxes (CSB) termination associated sequence (TAS) and origin of H-strand replication (O_H).

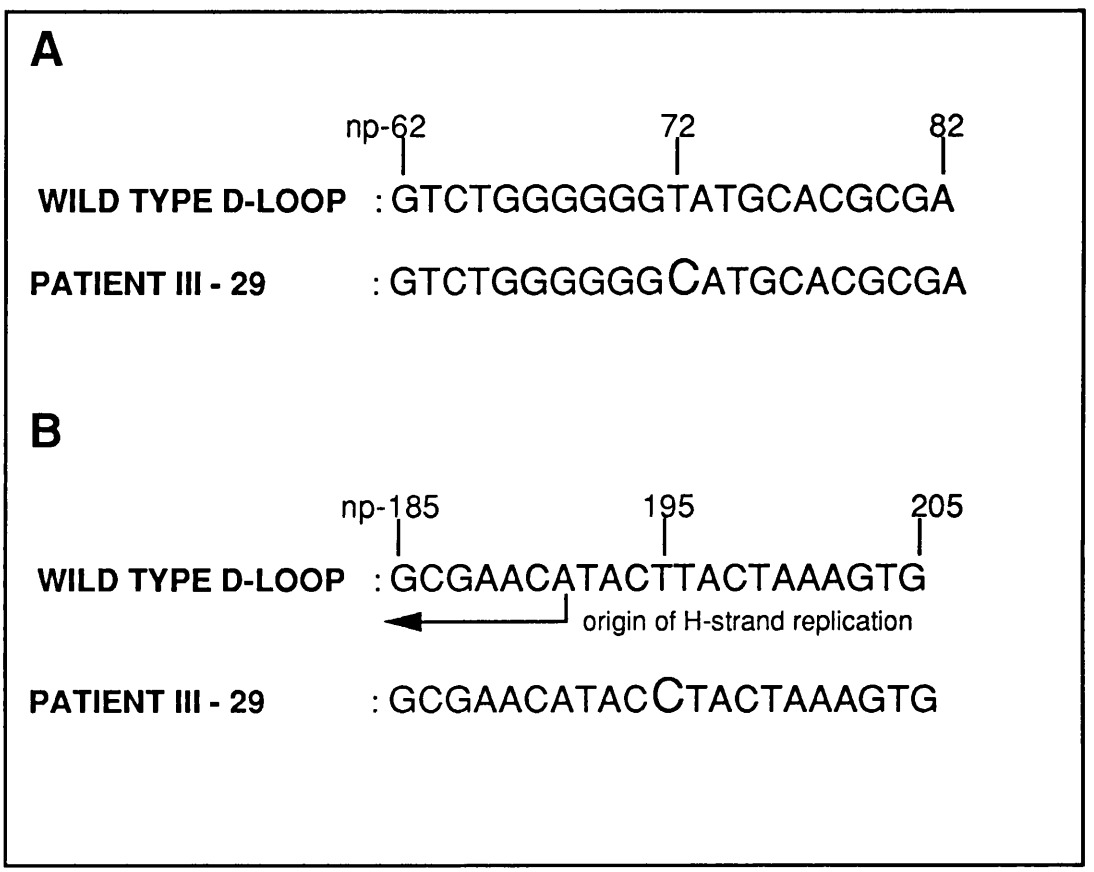


Figure 4:5 Illustrations of regions of mitochondrial genome around polymorphisms at np72 and np195.

A - showing 10np upstream and downstream of the polymorphism at np72

B - showing 10np upstream and downstream of the polymorphism at np195 indicating proximity to origin of H strand replication

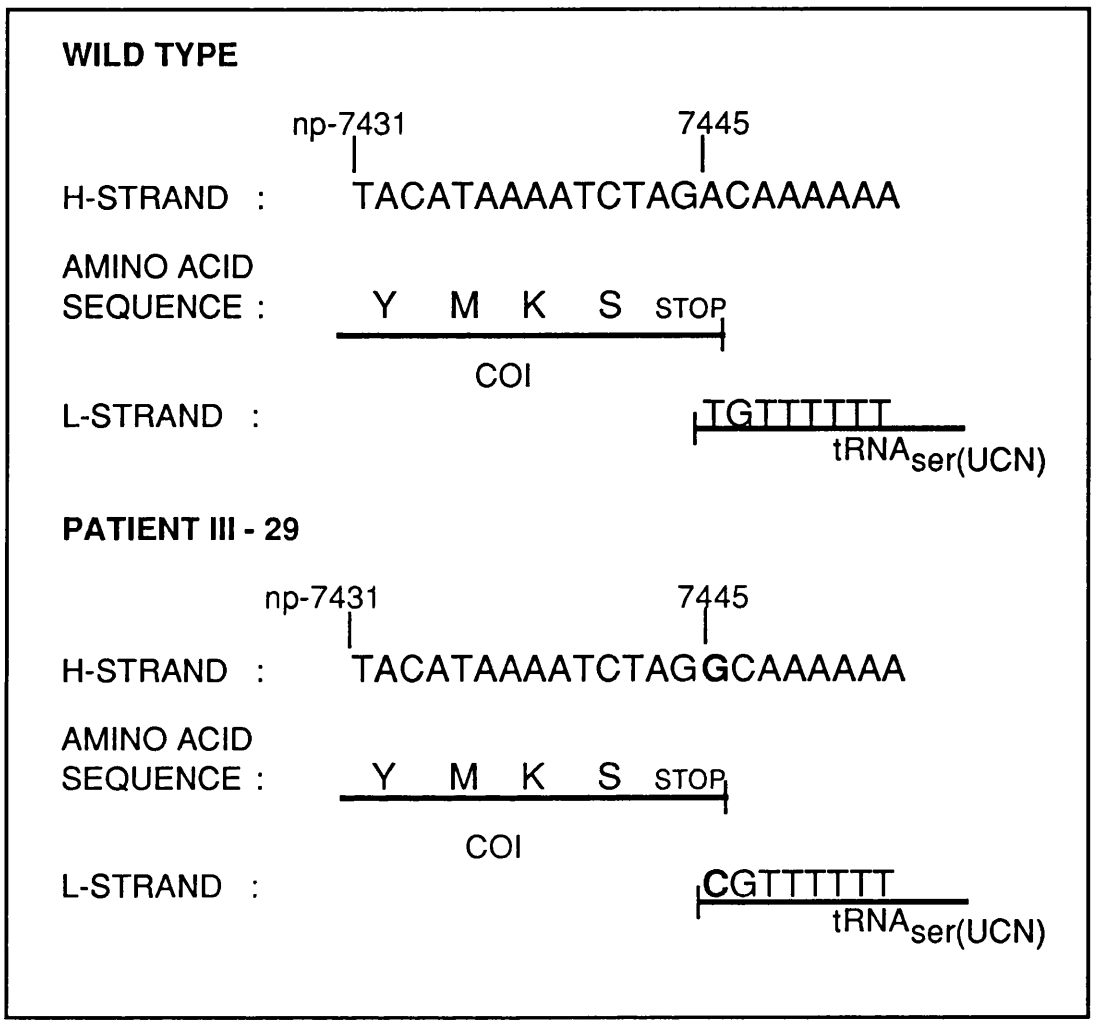


Figure 4:6 Upstream and downstream regions around the np 7445 mutation. The 7445 site has a function on both the H strand where it is the 3' nucleotide of the COI gene and the L strand where it is the extreme 3' nucleotide of the tRNAser(UCN) gene.

mutation. The polymorphism in the gene for cytochrome *b* at np 15110 causes an amino acid change: Alanine->Threonine. The effect of this nucleotide change on the amino acid sequence of the region immediately surrounding it is shown in Figure 4:7.

4:5 GENOTYPE OF MEMBERS OF THE PEDIGREE AT np 7445

Having found these polymorphisms in the index case other members of the pedigree were screened for the nucleotide change at np 7445. This was done in two ways: initially by restriction digestion using *Xba*I and, where doubt remained regarding the disruption of the site, direct sequencing was carried out.

4:5:1 Restriction fragment length polymorphism (RFLP) analysis

Blood samples were taken from a) 40 normal controls, by Dr Colin Reid, b) 24 non-syndromic deaf patients, including 1 with a maternal history of deafness, by Mr Guy Vernham; c) members of the pedigree living in Scotland, by Mr Guy Vernham; d) members of the pedigree resident in Australia, by Dr Patrick Rundle and colleagues. After preparation of DNA (see Chapter 2) PCR was carried out using as forward primer FR31/7178B and as reverse primer FR32/7840L (see Table 2:1:2) giving an amplified product of 662 np. Digestion of this amplified product with *Xba*I gives fragments of 400 np and 262 np when the site is intact.

In all normal controls and deaf subjects outside of the pedigree, as well as in individuals III-17,18,19 and 20, the fragment sizes predicted from the Cambridge sequence were found. In most maternally related members of the pedigree tested the amplified product was uncut. However, in a few family members there appeared low levels of the 400 np and 262 np fragments, whilst most of the DNA remained uncut. Figure 4:8 shows both situations - the samples where the amplified product is all intact (samples III-26 and III-28) and sample II-3 where low levels of restriction digest products are present.

This result could have two causes: heteroplasmy is a situation commonly found in mitochondrial diseases, the partial digestion could be the result of the presence of two mitochondrial DNA species in one individual one mutant, one normal. Alternatively the restriction digestion result could be due to contamination of a mutant DNA sample with minute quantities of amplified products from a normal individual. Fresh DNA samples were prepared from blood from the six pedigree members in which this phenomenon was observed and identical restriction digestion results obtained, consistent with heteroplasmy.

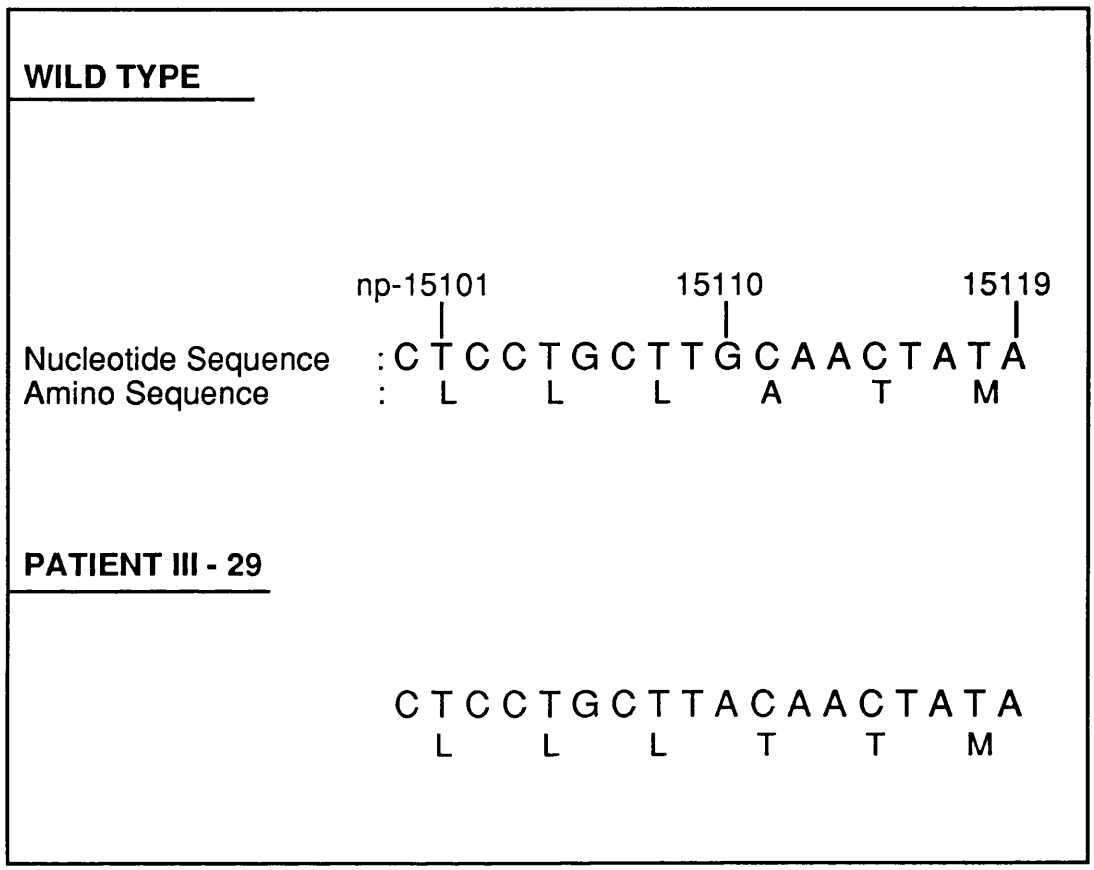


Figure 4:7 Nucleotide and amino acid sequences in region surrounding 15110 polymorphism. The G → A change at np 15110 found in the index case can be seen to change the codon from that for Alanine to that for Theonine.

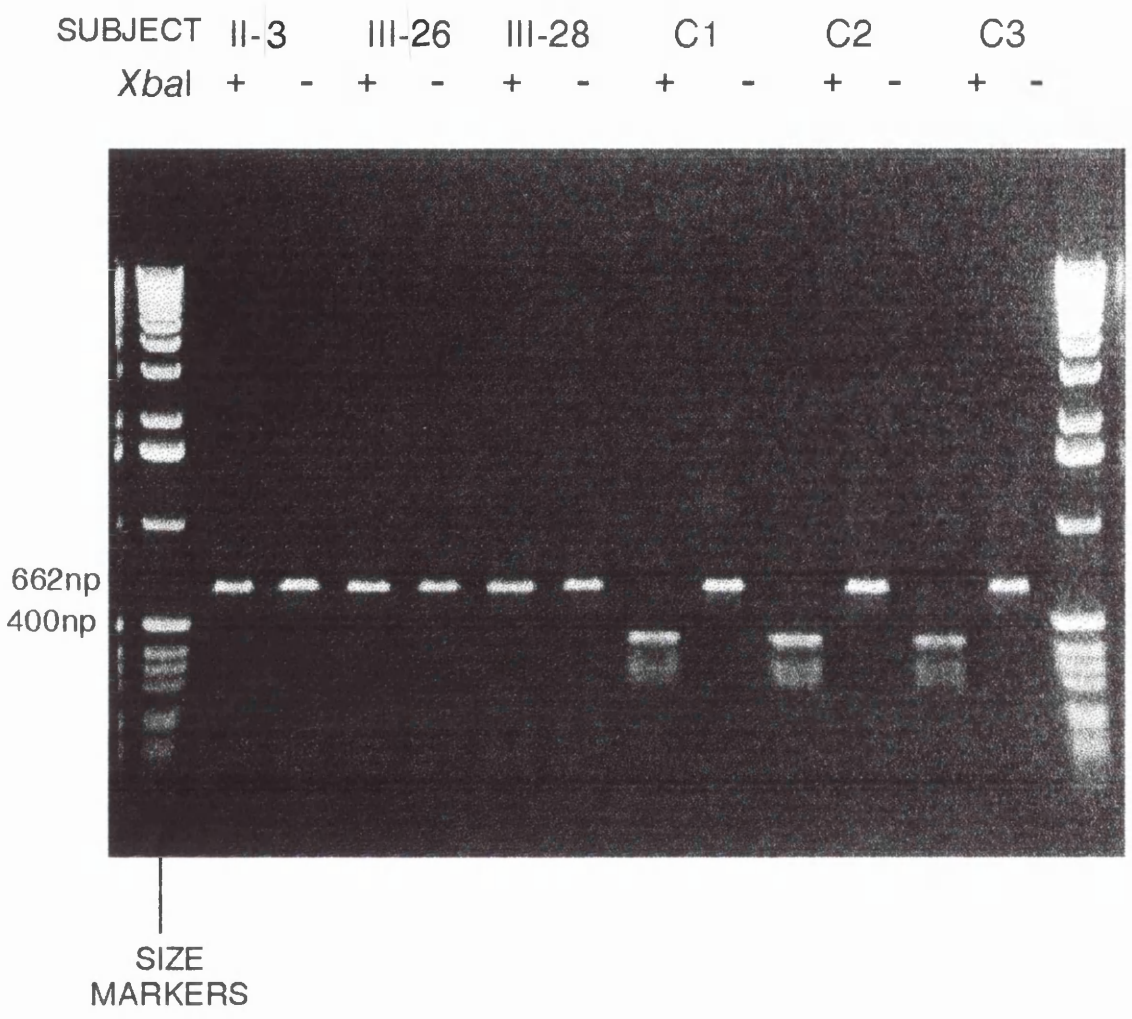


Figure 4:8 Restriction fragment length polymorphism analysis of 3 members of the pedigree and 3 controls. The 662np fragment is completely digested in the three control samples (C1, C2, C3) giving products of 400np and 262np. The sample from the two family members, III-26 and III-28, is undigested, indicating loss of the *Xba*I site. Sample II-3 shows partial digestion.

4:5:2 Sequence analysis at np 7445

As cautioned by Johns et al (1993), loss of a restriction site can be accounted for by a mutation at any of the nucleotide pairs within the site. In order to confirm that the base change causing loss of the *Xba*I site in all family members was the same as in the index case each available family DNA sample was sequenced. The 662 np fragment was amplified in multiple reactions, purified and sequenced using primer FR50/7567L for the L strand and FR49/7324H for the H strand.

Figure 4:9 shows the anti-sense strand sequence of a control and a member of the pedigree in which there had been complete lack of digestion of amplified product by *Xba*I. The homoplasmic T->C change at np 7445 can clearly be seen.

The *Xba*I site loss at np 7440 was reported by Brown et al in 1992 in two patients suffering from LHON. Sequencing of the region around this site showed the mutation to be a homoplasmic G->A at np 7444 in both LHON patients. Examination of 89 normals and a review of the literature by Brown et al showed that 6/595 controls had lost this *Xba*I site. (Horai et al, 1984; Harihara et al, 1988; Vilkki et al, 1989; Dionne et al, 1991; Ballinger et al, 1992). Brown et al sequenced only one of those six and found the same 7444 change. They made the assumption that the remaining five had the same change at this position.

Mitochondrial DNA from two of the remaining 5 control individuals were sequenced in the laboratory of Savontaus (personal communication) and were also found to have the np 7444 G->A change. DNA samples from two of the remaining three were donated to our laboratory by Drs Dionne and Skinner and sequenced: the 7444 change was confirmed in these two samples. DNA from the sixth control sample was not available from Japan. Combination of this data with that from the normal control samples screened in our study indicates the A->G change at np 7445 is not present in more than 600 controls.

Direct sequencing of amplified products from those family members in which traces of products of the *Xba*I restriction had been seen gave the result shown in Figure 4:10. At the np 7445 position in each of the six individuals there were two nucleotides, i.e. T and C. When the other strand was sequenced A and G were seen at position np 7445.

Since heteroplasmy of a mutation site is said by many to be the hallmark of its involvement in disease it was important to confirm whether these findings were a PCR artefact.

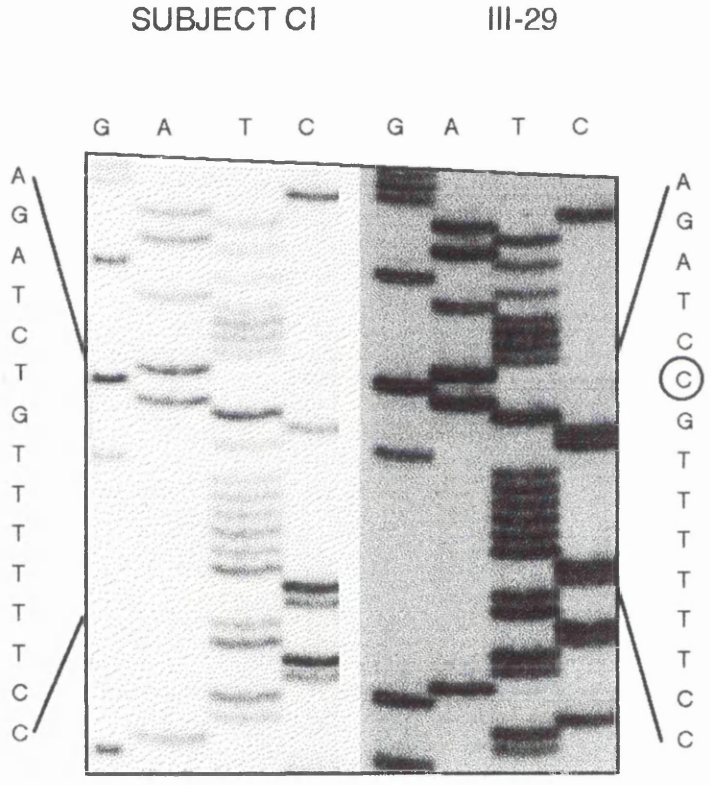


Figure 4:9 Direct sequencing across the COI/tRNAser(UCN) junction from patient III-29 and a normal control. Sequence shown is of the antisense strand and the nucleotide change indicated by a circle.

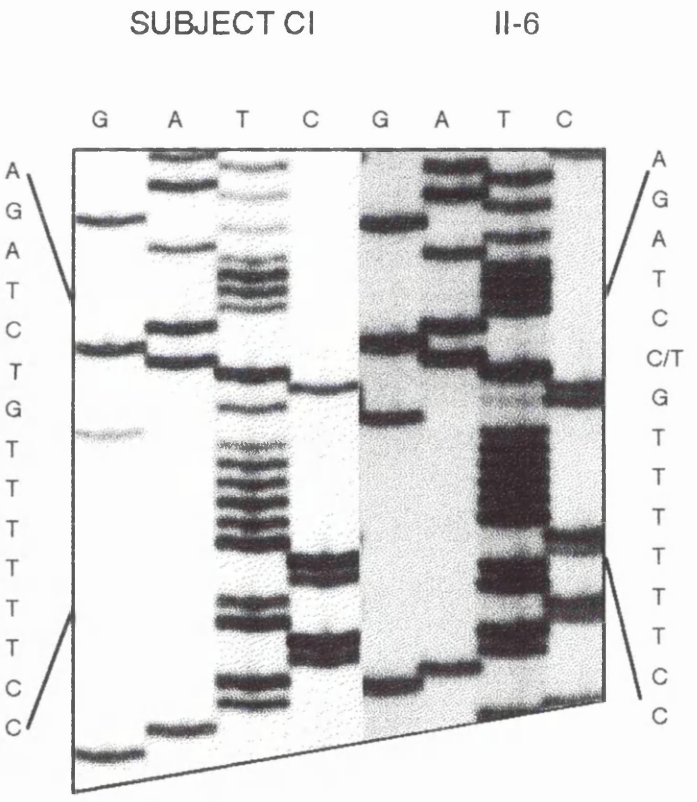


Figure 4:10 Sequencing of mt DNA across the COI/tRNAser(UCN) junction from normal control and patient II-6. The presence of both C and T at the np 7445 is clear in patient II-6

4:6 FURTHER INVESTIGATION OF HETEROPLASMY AT np 7445

It was possible to take two approaches to resolve the existence of heteroplasmy at np 7445 in some members of the pedigree: PCR carried out using a reverse primer downstream of FR32/7840L and Southern blotting following *Xba*I digestion of non- amplified mitochondrial DNA. The heteroplasmy question was resolved in this instance using the former approach.

Amplification was carried out using the same forward primer as before, FR31/7178B, and a reverse primer, (FR34/8562L), further downstream than that used previously (FR32/7840L). Direct sequencing of the biotinylated strand was carried out using FR50/7567H as an internal primer. If the heteroplasmy is real it should be seen at the same level as in the experiments described in Section 4:5. If, on the other hand, the apparent heteroplasmy is due to minute contamination of a DNA sample mutant at np 7445 with the 662 np PCR product from a normal control then this sequencing will show only a single nucleotide at this position. The apparently heteroplasmic samples were sequenced in this manner and only the A->G change found at np 7445, with no evidence of another nucleotide, at least within the limits of detection by DNA sequencing.

4:7 ANALYSIS OF MITOCHONDRIAL GENOTYPE AT np 15110 BY SSCP

The polymorphism found at np 15110 in the index case has not been reported in the literature. A change at this site is not detectable using an easily obtained restriction enzyme. An alternative, rapid method of screening a large number of samples was sought. The technique of SSCP is one in which PCR amplification products are electrophoresed through a non-denaturing polyacrylamide gel. Mobility shifts are observed depending on the structure of the single-stranded DNA (Orita et al, 1989). Amplification products should ideally be 200-300 np in length but even if the size is within this range not all point mutations can be detected using this technique. In order to screen for the 15110 polymorphism PCR was performed on control DNA from subjects who had already had the region surrounding np 15110 sequenced and been shown to be wild type at this site, in addition to using DNA from subject III-29 as template. The forward primer used was FR78/14973H and the reverse primer FR70/15218L giving an amplification product of 244 np.

A mobility shift on a silver-stained polyacrylamide gel was only found in the PCR product obtained using template DNA from the patient. The control samples all had a band at the same position which is different from that of the patient.

Having confirmed that the np 15110 polymorphism present in patient III-29 did give an observable mobility shift, the SSCP technique was used to screen 22 normal controls, 24 sporadic deaf patients and 10 members of the pedigree. In all experiments one member of the pedigree known to have the homoplasmic G->A at np 15110 was included. Figure 4:11 shows a typical silver-stained gel in which the PCR product from patient III-29 (number 31) migrates to a different position to that prepared using template DNA from normal controls (11,12,14 & 70) or sporadic deaf patients (4,5,10 & 48).

This particular mobiliy shift was only found when DNA from members of the pedigree was used as template, indicating that none of the other individuals tested had the A->G mutation at np 15110.

4:8 DISCUSSION

4:8:1 Inheritance of sensorineural hearing loss within the pedigree

Sensorineural deafness has previously been reported as the principal symptom in a number of pedigrees in which inheritance is apparently via the maternal line (Elverland & Torbergsen, 1991; Jaber et al, 1992; Prezant et al, 1993; Mutoni et al, 1992). Interactions of mitochondrial DNA with nuclear genes (Bu et al, 1993) or environmental agents (Hu et al, 1991) make the role of mitochondrial DNA mutations in the development of deafness difficult to define. Extensive sequence analysis of the mitochondrial DNA of the index case in the pedigree shown in Figure 4:1 was undertaken in order to detect any mutation which could be responsible for the phenotype.

Within this pedigree there is no evidence of paternal transmission of the defect; the inheritance pattern is compatible with maternal transmission. Although there are more females than males in the family in no case where a male could have transmitted the trait has he done so. Nevertheless it should be borne in mind that individual IV-1 is less than 2 years old and may yet develop symptoms. Maternal transmission often resembles the inheritance pattern of autosomal dominant except that the affected parent is always female. A test of purely maternal inheritance is therefore identification of any evidence of paternal transmission of a trait. In an attempt to create mathematical models for the study of mitochondrial inheritance Schork & Guo (1993) took a number of aspects of mitochondrial genetics into account including heteroplasmy, tissue specificity and interaction of nuclear gene products with mitochondrial gene products. Using a hypotheical pedigree they derived complicated models and tested strategies which they hope will help in the determination of mitochondrial inheritance. They do,

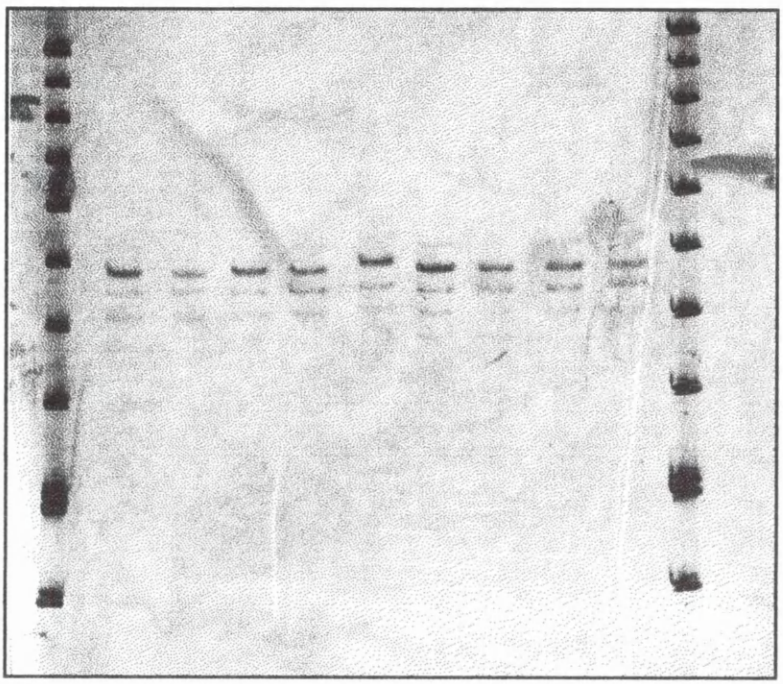


Figure 4:11 Single stranded conformational polymorphism (SSCP) analysis of 4 controls, numbers 11, 12, 14 and 70; 4 cases of sporadic sensorineural hearing loss, 4, 5,10 and 48 and the index case, 31. A product of 244 np was amplified using forward primer FR78/14973H and reverse primer FR70/15217L

however, acknowledge the potential problem of the range of phenotypic expression of mitochondrial DNA mutations.

Other modes of transmission cannot be completely excluded in this pedigree (e.g. autosomal dominant with incomplete penetrance). Many members of generation III are still children and since age of onset of deafness in the family ranges to 18 years it is possible that a number of them may develop deafness and thereby clarify the mode of inheritance. Given the present medical history and audiometric data, however, maternal inheritance of sensorineural hearing loss is most probable. The appearance in the pedigree of two maternally related females with unusual ophthalmological symptoms may also be attributable to a mitochondrial defect. The optic disc pallor observed in III-10 and III-15 is extremely rare but is a symptom also observed in the late acute stage of LHON (Nikoskelainen, 1985).

4:8:2 The pathological significance of the np 7445 mutation

The complete sequence of the mitochondrial DNA of patient III-29 revealed that the sequence deviated from the Cambridge sequence (Anderson et al 1981) at 13 nucleotide positions (Table 4:2). Nine of these deviations have been reported in normal controls by a number of different laboratories and they are thought to be either mistakes in the original sequence or relatively common polymorphisms. Four changes have not previously been reported: at np 72, np 195, np 7445 and np 15110. Of these the most interesting is the homoplasmic A->G at np 7445. This alteration disrupts an *Xba*I site at np 7440 which is at the junction of the COI and tRNAser(UCN) genes.

All maternally related members of the pedigree had the A->G change at np 7445. Initially six of these appeared to be heteroplasmic at this site. However, further investigations suggested that this was due to experimental contamination of DNA with minute amount of PCR products. This serves as a caution for such investigations: working practices should be such as to minimise the possibility of such contamination and any apparent heteroplasmy must be very thoroughly confirmed, using the methodology outlined in Section 4:6.

Precautions to minimise PCR product contamination of reactions should include setting up reactions in a laminar-flow cabinet. It should always be the case that PCR amplification product is not manipulated in the same room in which reactions are prepared. In addition micropipette tips containing filters should be used along with positive displacement micropipettes. At each new manipulation fresh gloves should be worn; discarded gloves, tubes and tips should all be

disposed of in a sealed bag which is put immediately into a waste bag for daily removal.

The pathological significance of the np 7445 mutation is supported by the fact that A-7445 is conserved in man's closest relatives, i.e. common chimpanzee, pigmy chimpanzee and gorilla (Horai et al, 1992). A T at this site is, however, not conserved down through the vertebrates: rat and mouse also have T but it is an A at this position in cows and G in *Xenopus* (Roe et al, 1985).

The np 7445 is assigned a function on both strands of mitochondrial DNA (see Figure 4:6) changing the stop codon of the COI gene on the H strand from AGA to AGG and altering the extreme 3' nucleotide of the tRNAser(UCN) on the L strand. The role of the mutation in contributing to the pathological phenotype of sensorineural deafness in this family, could involve either strand or both.

Regarding the effect of the mutation on COI, the alteration of the stop codon from AGA->AGG is thought to be silent as both are recognised as stop codons in human mitochondrial DNA. The mammalian mitochondrial genetic code has 4 termination codons: UAA, UAG, AGA and AGG (Cantatore & Saccone). Some genes have their termination codon created post-transcriptionally by poly-adenylation. In the universal genetic code AGA and AGG code for Arginine; these codons are not found within any mammalian mitochondrial gene, only at the 3' end of some genes. AGA has been identified as the termination codon for the human COI gene, while AGG has been identified in ND6 at the extreme 3' position (Anderson et al, 1981).

In LHON the G->A mutation at np 7444 changes the AGA stop codon to AAA which codes for lysine. Brown et al (1992) show an autoradiograph of an SDS-PAGE gel which they claim shows the COI polypeptide from a LHON patient, with the np 7444 mutation, migrating as a 37 kDa protein in contrast to that from normal controls migrating as 38 kDa. They attribute this faster migration to extension of the COI polypeptide by the 3 amino-acids Lysine, Glutamine, Lysine. In addition they report a 36% decrease in COX activity in one of the np 7444 positive LHON patients.

Their data would have been more convincing if both experiments had been carried out on both LHON patients they identified with the np 7444 mutation. In addition similar experiments performed using cells from the normal control identified as having the np 7444 mutation would help to clarify whether the results can really be attributed to the np 7444 mutation. One of the LHON patients was also reported to have the np 3460 mutation previously found to be

associated with LHON. Brown et al (1992) do not state which patient donated samples for each experiment. Obviously this is an important detail which has been omitted.

The decrease in COX activity of lymphocytes is surprising given the very specific phenotype of optic nerve damage in LHON. Similar studies in the deaf pedigree may give some insight into the effect of the np 7445 mutation on the activity of Cytochrome oxidase and its role, if any, in their deafness. This will be discussed further in Chapter 6.

4:8:3 The effect of the np 7445 mutation on $tRNA_{ser(UCN)}$

The change brought about by the 7445 mutation on the antisense strand is perhaps of more significance. All tRNAs are thought to have a similar three dimensional structure, the typical "cloverleaf" as illustrated in Figure 4:12. The precise structure of human mitochondrial tRNAser(UCN) has not been determined directly. In human mitochondria the 3' terminal CCA of tRNAs is not encoded in the DNA but added post transcriptionally (Attardi 1985). The change at np 7445 will therefore be at the 4th nucleotide from the 3' end of the mature tRNAser(UCN). In *E. coli*, yeast and rat the base at this position has been reported as invariant in tRNAs specific for a given amino acid (Crothers et al, 1972). This position was given the term "discriminator" residue and is proposed to function as a positive identity element, which the amino-acyl synthetase specific for each amino acid recognises directly. The discriminator residue is believed to play an important role in tRNAser in *E. Coli* (Normanly & Abelson, 1988). ***In vitro*** studies indicated, however, that it is not important in bovine mitochondrial tRNAser(AGY) where base replacements at the discriminator site did not affect the addition of the serine residue to the tRNA.

A relatively small number of nucleotides are important in the specificity of amino- acylation; eight identity elements have been identified in *E coli*. Unlike most tRNAs in *E.Coli* (with the exception of $tRNA_{ala}$) tRNAser does not use the anti-codon as the major recognition site for amino acylation.

Mis-acylations of tRNAs are relatively easy to induce using special reaction conditions (Schimmel, 1987): this implies that subtle changes in tRNA structure and/or amino acyl synthetase are sufficient to induce mis recognition and lead to mis- charging of the tRNA. Sensorineural hearing loss is a very specific phenotype in the family under study. Consequently if there is mis charging of tRNAser(UCN) it must be extremely tissue-specific; the tRNAser must be able to function normally in other tissues. Tissue-specific mis-charging could arise if

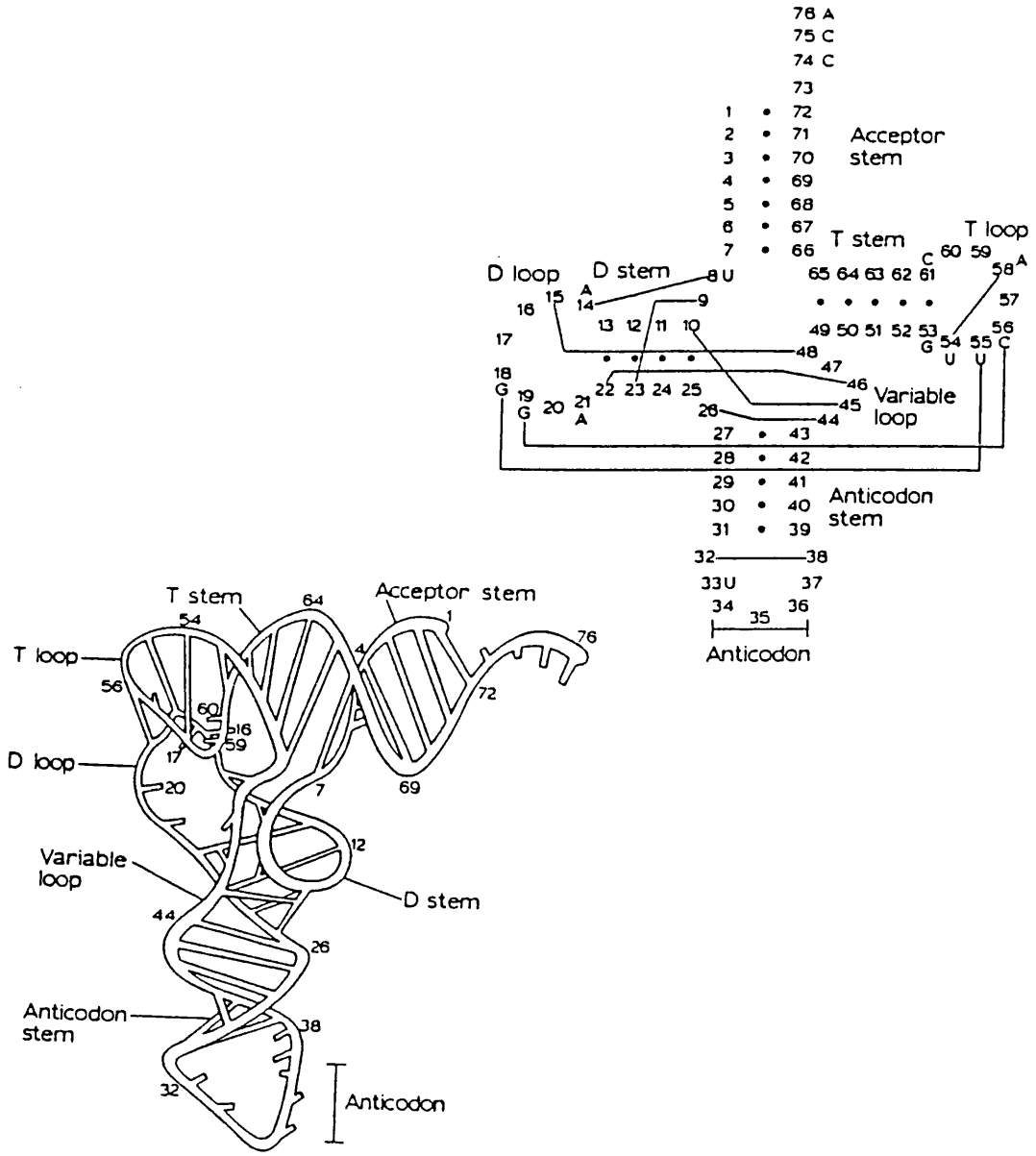


Figure 4:12 Secondary and Tertiary structure of a stereotypical tRNA. Typical "cloverleaf" secondary structure and L model tertiary structure of tRNA. Dots indicate base pairing. From Normanly & Abelson (1989).

4:8:3 cont.

The work of Yokogawa et al. (1991) suggested a novel cloverleaf structure for bovine mitochondrial tRNAser(UCN). They conclude from their results that this tRNA does not have two U-U mismatches at the top of the acceptor stem as has previously been thought. In addition they propose that in mouse mitochondrial tRNAser(UCN) the exclusion of one nucleotide at the $3^{'}$ end would allow a cloverleaf structure similar to that found in bovine tRNAser(UCN).

If such a structure is proposed for human tRNAser(UCN) then the nucleotide at position 7445 in the gene would not be represented in the tRNA . Until the precise three dimensional structure of human mitochondrial tRNAser(UCN) is determined the involvement of the 7445 nucleotide in the tRNA will be the subject of debate.

another less efficient isoform of the specific amino acylsynthetase is present in the nerve cells of the vestibular cochlea (8th cranial nerve) in those developing deafness.

The mitochondrial amino acyl tRNA synthetases are encoded on the nuclear DNA, translated in the cytoplasm and imported into the mitochondria (Ueda et al, 1992). If there are tissue-specific isoforms of amino-acyl-synthetases then there are a number of mechanisms which could possibly account for tissue specific mis-translation of mitochondrial proteins. The charging efficiency may be different between isoforms and it is possible to envisage a situation where an equilibrium between isoforms of different efficiencies could alter in different tissues. Some isoforms could also be less specific in their charging specificity, allowing a tRNA to be charged with an amino acid with which it is not usually associated. A defect in a nuclear gene resulting either in a less efficient isoform aminoacyl synthetase or in a less efficient import into mitochondria could work in concert with the mitochondrial mutation to give rise to the pathological phenotype.

There are obvious parallels which can be drawn between the molecular mechanisms proposed in the development of AAID associated with the np 1555 mutation, described in Chapter 3, and those described here. It seems unlikely to be only a coincidence that the mitochondrial mutations identified in both situations map to regions of the mitochondrial genome which are involved in translation. The relationship between the two will be further discussed in Chapter 7.

4:8:4 Possible RNA processing effects of the np 7445 mutation

An alternative hypothesis is that interference with RNA processing caused by the change at np 7445 could interfere with maturation of either COI mRNA, tRNAser(UCN) or both. The polycistronic transcripts of mitochondrial DNA are processed by mechanisms which involve the recognition of junctions between the tRNAs and the mRNAs or rRNAs (Maram & van Tuyle, 1987). These mechanisms are thought to be dependent on the stereotypic tertiary structures of tRNA segments in the primary transcript and are assumed to involve the recognition of boundaries which can be recognised by endonucleases. Processing at the 5' terminus of tRNAs has been shown to be carried out by RNAseP or RNase P-like enzymes in prokaryotes (Kole & Altman, 1982), yeast mitochondria (Hollingsworth & Martin, 1986) and HeLa cell mitochondria (Doerson et al, 1985). In addition Maram & van Tuyle (1987) demonstrated that, in rat liver mitochondrial extracts, 5' processing of the *E. coli* tRNAtyr was obligatory before 3' processing could take place.

There have been a number of mutations in mitochondrial tRNAs associated with disease phenotypes which are thought to be due to defects in mitochondrial RNA processing. An A->G change at np 3243 has been found in MELAS patients, this is located in the tRNAleu(UUR) gene. King et al (1992) identified an abnormally large amount of an RNA intermediate in cells containing high percentages of mitochondrial DNA mutant at the 3243 site. This intermediate was named RNA19 by extension of the nomenclature for the human mitochondrial RNA species. It was shown, by Northern analysis, to consist of a polycistronic transcript of 16SRNA, tRNALeu and ND1. The 3243 mutation is thought to result in an RNA processing defect and this could be involved in the severe respiratory chain deficiencies observed in MELAS. What that involvement is has not been determined, King et al (1992) suggested that the np 3243 mutation causes a number of distinct effects which cumulatively result in the MELAS phenotype.

Bindoff et al (1993) found a similar processing deficiency in a patient suffering from a mitochondrial myopathy who had a different mutation in tRNALeu; at np 3302. In this case the mutation is at the 3' end of the tRNA, in the aminoacyl stem. Northern analysis revealed that, in addition to RNA19, there were other RNA intermediates present in skeletal muscle and alternative ones in skin fibroblasts. Results were consistent with processing occurring at the 3' end of the tRNALeu in fibroblasts, and the 5' end in skeletal muscle. Fibroblasts from this patient had no biochemical defect while skeletal muscle showed severe loss of Complex I activity, in keeping with the phenotype of myopathy.

The nucleotide change at np 7445 could possibly result in an RNA processing defect. The extremely tissue specific phenotype may be due to tissue specific processing differences. Alteration to the COI transcript resulting from abnormal processing could give an altered amount or structure of the COI subunit of the Cytochrome *c* oxidase complex.

Human Cytochrome *c* oxidase has three mitochondrially encoded subunits and ten nuclear encoded subunits (Capaldi, 1990). The three mitochondrially encoded subunits which are homologous to the three subunits of the prokaryotic cytochrome oxidase, are essential for electron transport and proton pumping. The ten nuclear encoded subunits are thought to be regulatory or may have a "scaffolding" role within the mitochondrial inner membrane. Several of the nuclear encoded subunits are present in two or more tissue-specific isoforms (Capaldi et al, 1988). The 7445 mutation in this family may therefore result in an altered COI polypeptide which can interact normally with isoforms of nuclear encoded subunits present in most tissues. In contrast a different isoform present

in the auditory neurons may interact with the altered COI in such a way to cause decreased activity of the cytochrome oxidase complex. Neuronal tissue is extremely sensitive to changes in oxidative phosphorylation (Shoffner & Wallace, 1990), such a decrease in the 8th cranial nerve could give rise to sensorineural hearing loss.

When more than one isoform of an enzyme exist there are sometimes different ratios of isoforms in different tissue. For example, the adenine nucleotide translocator (ANT) has three isoforms which are present in varying ratios in different tissues (Stepien et al, 1992). A tissue specific alteration in the ratio of COX subunit isoforms would be consistent with the slow but steady progression of sensorineural hearing loss in this family.

4:8:5 Pathological roles of the other deviations from the Cambridge Sequence

Pathological roles for the remaining three polymorphisms, found in the index case, cannot be excluded but seem less likely. Positions np 72 and np 195 are in the hyper- variable segment of the D-loop. Neither site is within a binding site for the mitochondrial transcription factor mtTFI (Parisi & Clayton, 1991); a conserved sequence box or within the termination associated sequence, TAS (Foran et al, 1988). These two positions within the D-loop have not been assigned a function although np 195 lies close to the origin of replication of the H strand (see Figure 4:5). Both are conserved in chimp, pigmy chimp, gorilla and orangutang. Their potential role in the pathology of deafness in this family is unknown, and could only be clarified by finding these mutations associated with other cases of sensorineural hearing loss.

The change at np 15110 in Cytochrome *b* changes an Alanine residue to a Threonine; this is normally regarded as a conservative amino acid change. However, as Figure 4:13 shows, the amino acid encoded at this position is not highly conserved. Although it is Alanine in rat and mouse, it is Valine in Sea urchin (Jacobs et al, 1988); Leucine in *Xenopus* (Roe et al, 1985) and *Drosophila* (Clary & Wolstenholme, 1985) and Threonine in cow (Anderson et al, 1982). Screening of more than 40 individuals for this polymorphism using SSCP has indicated that it is not a common polymorphism in the Scottish population. The same mobility shift was seen in all maternally related members of the pedigree tested, those whose mitochondrial DNA was sequenced at this position had the same homoplasmic G->A change at np 15110. The possibility that this mutation is involved in the development of deafness cannot be excluded, as with the mutations in the D-loop, but seems unlikely.

Human (HeLa)	G	I	I	L	L	L	A	T	M	A	T	A	F	M	G
Patient III-29	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.
Rat	.	.	.	.	.	F	.	V	.	.	.	.	.	.	.
Mouse	.	F	L	.	.	F	.	V	.	.	.	.	.	.	.
Sea Urchin	.	V	.	.	F	.	V	.	I	L	.	.	.	.	.
Xenopus	.	V	.	.	.	F	L	V	.	.	.	.	.	V	.
Bovine	.	V	.	.	.	.	T	V	.	.	.	.	.	.	.
Drosophila	.	V	.	I	.	F	L	V	.	G	.	.	.	.	.

Figure 4:13 Amino acid sequence of region of Cytochrome *b* from the amino acid 116 to 130. Human sequence of HeLa cells is aligned with the counter part in Patient III-29 and rat, mouse, sea urchin, *Xenopus*, cow and *Drosophila*. The dots represent identity with the Human Hela cells amino acid sequence at that position. The boxed area shows the lack of conservation of the amino acid at position 122 which is that affected by the nucleotide change at np 15110 in Patient III-29.

The homoplasmic change at np 7445 remains, therefore, the most likely candidate mutation to have a causative role in the sensorineural loss observed. However, a cumulative effect of the four mutations found cannot be excluded. In LHON a large number of mutations of mitochondrial DNA have now been reported and in some patients two or more occur together. It has been suggested (Brown et al, 1992; Johns et al, 1991; MacKey & Howell, 1992) that the clinical manifestations of LHON can be the result of a number of mitochondrial DNA mutations acting synergistically resulting in an overall decrease in mitochondrial oxidative phosphorylation in specific tissues. In a similar manner a disease haplotype may explain the relationship of the mitochondrial genotype with the phenotype of sensorineural hearing loss in this family.

The recent report of an additional family suffering from sensorineural hearing loss having the 7445 A->G change (N. Fischel-Ghodsian, personal communication) provides additional evidence that this mutation is involved in the aetiology of maternally inherited deafness. This family is based in New Zealand and is unrelated, as far as we know, to the pedigree described in Section 4:1. The hearing loss is more severe and the penetrance of the defect higher than our pedigree. Sequencing of the mitochondrial genome has revealed none of the polymorphisms unique to our pedigree. This evidence suggests that the np 7445 mutation has arisen independently in the two families and is strongly indicative of its involvement in the development in sensorineural hearing loss in these pedigrees.

However, some other factor must be implicated in the development of deafness as all maternally related members of the pedigree in Figure 4:1 have the 7445 mutation, but not all are deaf. Differences in nuclear background; differential tissue expression of a nuclear gene product; viral infection; environmental factors are all candidates for involvement in the development of deafness.

The possible role of viral infection in the development of deafness in the pedigree was briefly investigated in the process of interviewing patients. Individual III-6 had rubella just before he developed a hearing problem and II-7 had measles as a very young child. Other members of the family also had measles, chicken pox and mumps but in all cases they (or their Mother) could not remember the exact sequence of events. Most seemed to remember that the deafness had developed prior to the viral infection. This is obviously an area worthy of more detailed investigation, particularly in any other families identified.

The only environmental factor considered in this study was cigarette smoking. No relationship was found between those who smoke, had smoked or had a parent who smoked and the development of deafness.

Further investigation of the effect of the mitochondrial genotype found in the index case of this pedigree on cellular biochemical and molecular mechanism was undertaken. The results of this study are presented and discussed in Chapter 6.

CHAPTER 5 INVESTIGATION OF THE MITOCHONDRIAL DNA SEQUENCE IN A PATIENT SUFFERING FROM LHON AND SENSORINEURAL HEARING LOSS

5:1 MEDICAL HISTORY OF PATIENT SH

The patient, known as SH throughout the study, presented originally with bilateral optic neuritis in 1978 when he was aged 26. He was diagnosed as having Leber's Hereditary Optic Neuropathy (LHON) by Dr Bronte-Stuart at the Department of Ophthalmology, Western Infirmary, Glasgow. SH has been registered blind since 1983 and now has a guide dog. Apart from an unexplained episode of apparent paralysis at the age of 15 SH had no other neurological symptoms until he developed hearing loss in 1988. Mr Andreas Nicolaides investigated this at the Victoria Infirmary, Glasgow where the staff of the ENT Department carried out audiometry and identified a mild sensorineural hearing loss. Figure 5:1 shows an audiometric trace for SH obtained in 1992, showing bilateral loss with a pure-tone average of 33 dB. The pattern of the trace indicates that there is no significantly greater loss at high frequencies, a pattern unlike that observed in the pedigree described in Chapter 4.

5:2 FAMILY HISTORY OF PATIENT SH

This study was initiated in 1991 on the basis of information, provided by Mr Nicolaides, on the family history. This was that SH had two maternal relatives who also suffered from sensorineural hearing loss: a maternal uncle and aunt. When contacted and interviewed, by me, in 1993 SH was found to have a different family history. His father (now deceased) suffered from glaucoma and had become deaf, but this was probably the result of an industrial accident. A maternal uncle has LHON and a maternal aunt has very poor eyesight which has never been fully investigated; neither is deaf.

The pedigree of SH as it is now known is given in Figure 5:2. Individuals I-4, I-5 and I-6 are all believed to suffer from deafness of undefined type and severity; I-2 suffers from LHON, and I-1 has very poor eyesight. The brother of SH, number II-7 died of a heart attack in his 40s, having been identified as having very high cholesterol. As far as SH is aware no member of the family suffers from diabetes, epilepsy or muscle weakness.

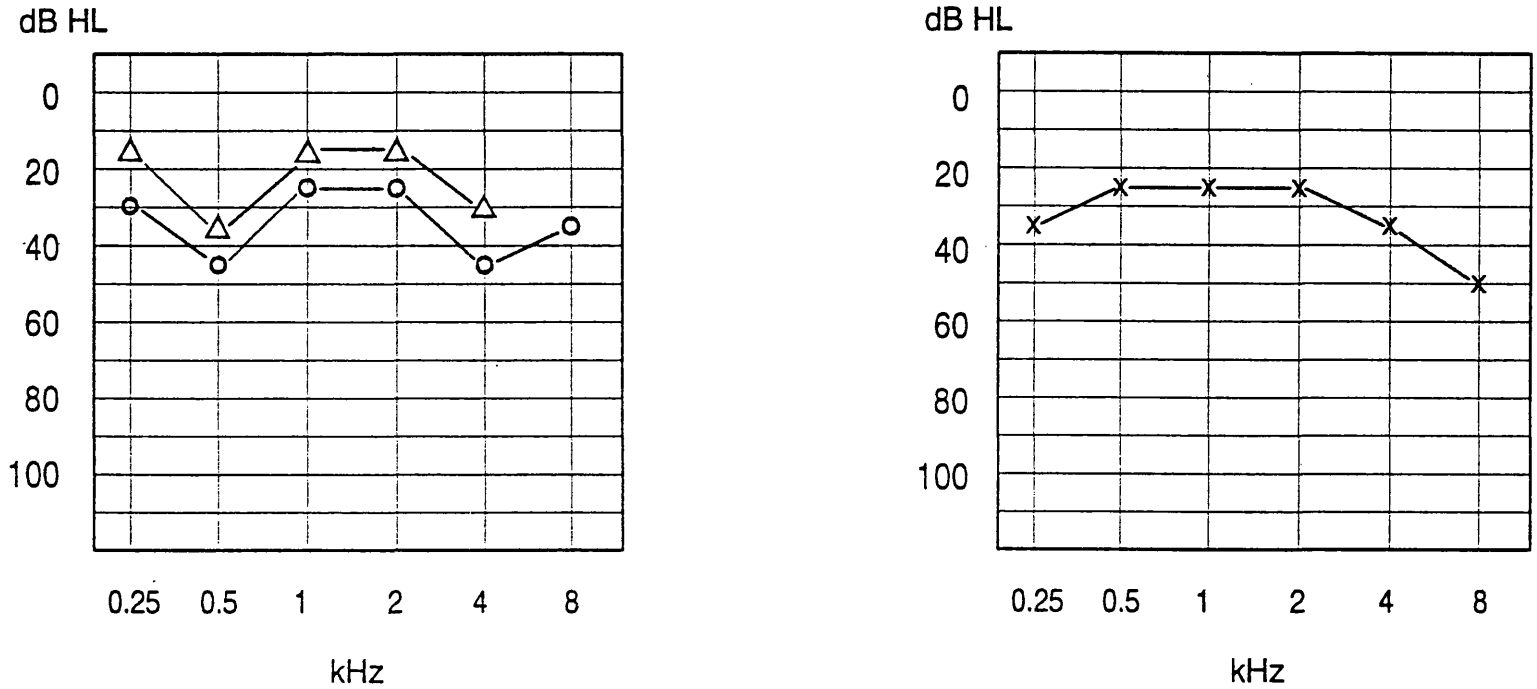


Figure 5:1 Audiometric trace for patient SH showing a pure tone average of 33 db.

Δ Right Bone Coductance
0 Right Air Conductance
X Left Air Conductance

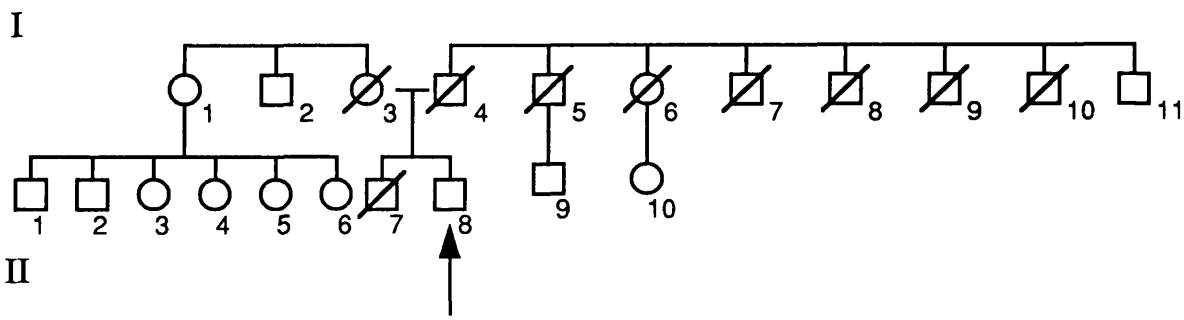


Figure 5:2 Pedigree of SH. Patient SH is number II8 indicated by an arrow.

5:3 RFLP ANALYSIS

Based on the information provided in 1991 it was possible that the deafness developed by SH was caused by mitochondrial DNA mutation. The combination of LHON with sensorineural hearing loss was unusual and a sequencing project was undertaken, the aim of which was to establish the sequence of the entire mitochondrial genome of SH after initially screening for previously identified RFLPs associated with LHON.

5:3:1 Creation of a permanent cell-line from the blood of SH

A lymphoblastoid cell-line was created by Epstein-Barr transformation of lymphocytes from the blood of SH. This was carried out at Yorkhill Hospital, Glasgow by Dr Mair Crouch under the direction of Dr Elizabeth Boyd in 1990. DNA was extracted according to the method given in Chapter 2:4, by Josephine Anderson, and stored at -20°C until required.

5:3:2 RFLP analysis of the "classical" LHON site

DNA was prepared from a blood sample donated by SH, using the method described in Chapter 2:4 . This, and the DNA extracted from tissue culture cells, was used as template in PCR reations with forward and reverse primers which create a product containing the SfaNI site encompasing np 11778. Primers L1/11688B and L2/11914L create a 226 np fragment; the products of the SfaNI restriction are 136 np and 90 np if the site is intact as shown in Figure 5:3. The amplified product using both DNA from blood and DNA from the cell-line could not be cut with SfaNI; the site had been lost in the patient SH, and the loss was homoplasmic.

5:3:3 RFLP analysis at sites previously associated with LHON and MELAS

PCR amplification products, using DNA from SH as template, were screened for RFLP sites at 3243, 3460, 4160, 7440 and 13708 as described in Chapter 3: . No change from the Cambridge sequence was found at any of these sites.

5:4 IDENTIFICATION OF SEQUENCE CHANGE RESULTING IN THE LOSS OF THE SfaNI SITE

Since a deviation from the Cambridge sequence at any of the 5 nucleotides involved in the SfaNI site at 11777 can result in its loss, the precise nucleotide that had been altered had to be determined by direct sequencing of PCR products.

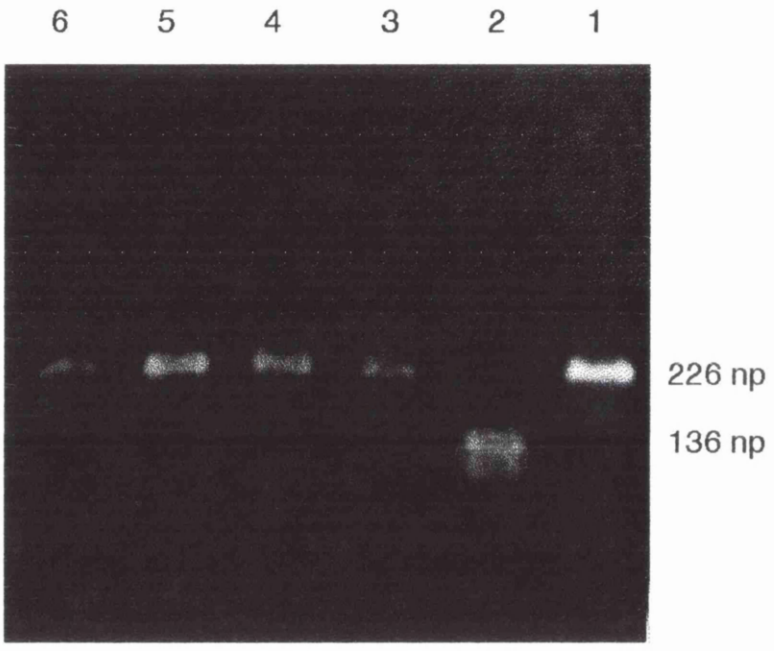


Figure 5:3 Restriction digestion of PCR product by SfaNI. Primers L1/11688B and L2/11914L were used to amplify a 226np fragment. Lanes 1, 3 and 5 are undigested products. Lanes 2, 4 and 6 are digested products from a normal control (1&2) DNA extracted from the SH cell-line (3&4) and DNA prepared from the blood of SH (5&6).

Using SH cell-line DNA as template, PCR was performed using primers L1/11688B and FR44/12553L. After purification and separation of strands (see Chapter 2:7&2:9) the + Bio strand was sequenced using primer L2/11914L. Figure 5:4 shows the antisense strand of SH alongside that of a normal control. The homoplasmic T->C (which is G->A on the sense strand) can be seen at np11778 in patient SH. Thus SH does have the "classical" LHON mutation in his mitochondrial DNA.

5:5 THE COMPLETE NUCLEOTIDE SEQUENCE OF THE MITOCHONDRIAL DNA OF SH

The entire mitochondrial sequence of patient SH was determined using direct sequencing of purified PCR products as described in Chapter 2:10. Where a difference from the "Cambridge" sequence was found it was confirmed by sequencing the other standard using, as template, DNA extracted from blood.

Table 5:1 summaries the differences found; 11/17 of these have been previously identified and are thought to be mistakes in the "Cambridge" sequence or polymorphisms. In addition to the LHON mutation at np 11778, 5 previously unreported deviations from the Cambridge sequence were found. Two in the D-loop at np 152 and np 16509 and three nucleotide changes causing no alteration in the amino acid, at np 4793 in ND2, np 8706 in ATPase 6 and np 15530 in Cyt *b*.

5:6 DISCUSSION

5:6:1 Mitochondrial DNA mutations associated with LHON

Leber's Hereditary Optic Neuropathy (LHON) is one of the most well documented mitochondrial diseases, as outlined in Chapter 1. Since the identification of the so-called "classical" mutation at np 11778 by Wallace et al in 1988, a large number of mitochondrial DNA mutations have been identified which are associated to a greater or lesser degree with the development of LHON. The mutations are now divided into two classes, primary and secondary.

Huoponen et al (1993) define a primary LHON mutation as one which fulfils the following criteria:

i. it has never been found in a normal control
ii. it alters an otherwise highly conserved amio acid
iii. it is associated with a measurable drop in oxidative phosphorylation

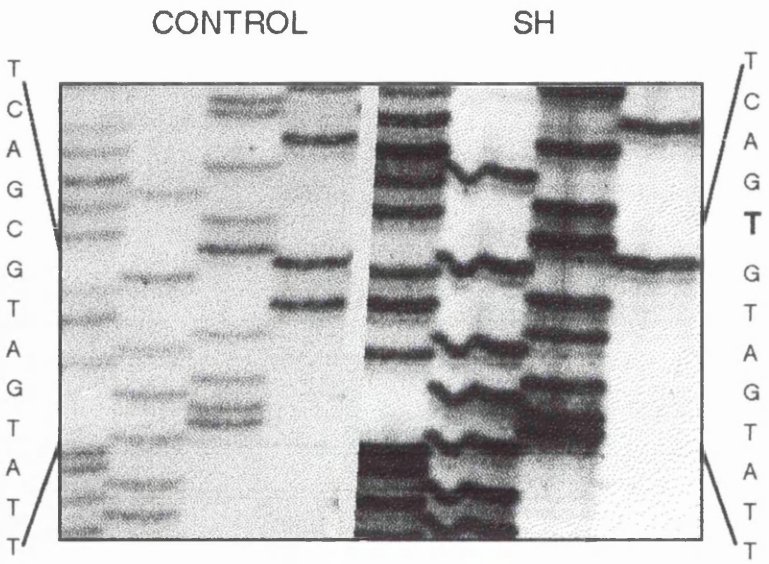


Figure 5:4 Sequence at np 11778 is SH and a normal control. PCR product amplified using L1/11688B and FR44/12553L. The antisense strand is shown and the C→T at np 11778 in SH indicated in bold.

Gene	Coordinate (np)	Change on sense strand	Amino acid substitution	Previously reported?
D-loop	152	T->C	-	No
12S rRNA	750	A->G	-	Yes Prezant et al (1993)
	1438	A->G	-	Yes Marzuki et al (1992)
ND1	3423	G->T	Silent	Yes Howell et al (1992)
ND2	4796	A->G	Silent	Yes Howell et al (1992)
	4793	A->G	Silent	No
	4985	G->A	Silent	Yes Howell et al (1992)
ATPase 6	8706	A->G	Silent	No
ND4	11335	T->C	Silent	Yes Howell et al (1992)
	11778	G->A	Arg->His	Yes Wallace et al (1988)
ND6	14199	G->T	Pro->Thr	Yes Howell et al (1992)
	14365	G->C	Silent	Yes Howell et al (1992)
	14368	G->C	Phe->Leu	Yes Howell et al (1992)
cytochrome *b*	14766	T->C	Ile->Thr	Yes Lertrit et al (1992)
	15326	A->G	Thr->Ala	Yes Marzuki et al (1992)
	15530	T->C	Silent	No
D-loop	16509	T->C	-	No

Table 5:1 Deviations from Cambridge Sequence of mtDNA from patient SH

Two mutations are universally assigned as primary: the A->G at np 11778 in ND4 and the G->A at np 3460 in ND1. Two others are the subject of debate: T->C at np 4160 in ND1 and T->C at np 14484 in ND6. The mutation at np 4160 has only ever been identified in one Australian family (Howell et al, 1991), while that at np 14484 has almost always been found in combination with some other secondary mutation (Johns et al, 1992) and is associated with partial recovery of vision.

Secondary mutations are those whose association with LHON is uncertain as they also occur in normal controls although at a lower frequency. In addition to the secondary LHON mutations given in Table 1:2 others have now been reported: a G->A at np 14459 in ND6 identified in one five generation Hispanic family with LHON and early onset dystonia (Jun et al, 1994). At the meeting of the European Society of Human Genetics, 1994, de Vries et al reported two further mutations in a large Dutch family at np11696 in ND4 and npl 4596 in ND6. The A->G at np 11696 was heteroplasmic, while the T->A at npl4596 was homoplasmic. The phenotype was variable, some members of the family having spastic dystonia and LHON and others having only one symptom.

In all LHON pedigrees studied not all individuals who have the mutations necessarily show symptoms of disease (Gerbitz et al, 1994). It follows that, in a similar manner to the deaf pedigree described in Chapter 4, other factors must be involved in the development of the disease phenotype.

5:6:2 Deviations from the Cambridge sequence found in SH

The patient investigated in this study was diagnosed as having LHON in 1978 at the age of 26. He received treatment of Cystine and Neo-cytamen from 1979 with no improvement by 1983 when the treatment was greatly reduced. At diagnosis in 1978 SH was advised to stop smoking cigarettes and changed to pipe smoking. Clinicians at the Western Infirmary, Glasgow recognised classical symptoms of LHON; optic disc pallor and centrocecal scotoma. After his development of deafness in 1988 and in view of what we believed to be a maternal inheritance of deafness in addition to LHON this large scale sequencing project was undertaken.

The presence in SH of the most common mitochondrial DNA mutation associated with LHON was found at np 11778 by means of restriction enzyme digestion using SfaNI (Figure 5:3) and direct sequencing (Figure 5:4).

The entire mitochondrial DNA sequence was subsequently established by means of PCR and direct sequencing. There are, in total, 17 nucleotide substitution

present (Table 5:1). There is a suggestions that LHON patients have more polymorphisms than normals (Savontaus - personal communciation), the implication being that there is an inherently higher mitochondrial DNA mutation rate in LHON patients. This is difficult to substantiate as the mitochondrial DNA of only a few LHON patients have sequenced in their entirity. Huoponen et al (1993) found 27 nucleotide differences in 23 Finnish LHON families but did not report individual sequences and the number of polymorphisms in each patient.

Two of the 5 deviations, from the "Cambridge" sequence found in SH, which have not been previously identified, lie within the hypervariable D-loop: T->C at np152 and T->C at np 16509. Neither site has been assigned a function; as can be seen from Figure 5:5. Neither lies within a conserved sequence box, a termination associated sequence, the origin of H-strand replication or either the light or heavy-strand promoters. The significance of mutations at these sites is unknown, but unlikely to be of importance.

The remaining 3 differences at np 4793, np 8706 and np 15530 result in silent amino acid alterations in ND2, ATPase 6 and Cyt *b* polypeptides respectively.

There are therefore no deviations from the established human mitochondrial sequence, apart from that at np 11778 in patient SH, which are obviously involved in the development of deafness. It is possible that the sensorineural deafness in SH arises via some unknown mechanism as a result of the specific pattern of mitochondrial DNA polymorphisms present. Screening of normal controls for each of the 4 previously undocumented differences would reveal whether or not they are simply rare polymorphisms. This could be done rapidly by SSCP (see Chapter 4) or the ARMS technique (Ferrie et al, 1992). The recent development of a PCR amplification technique which uses only 30 µl of blood or a single tissue culture cell (Erickson & Castora, 1993) will be an aid in these studies.

The combination of symptoms of optic atrophy and deafness in addition to diabetes insipidus, diabetes mellitus and sometimes additional symptoms such as epilepsy have now been recognised as Wolfram's Syndrome also known by the acronym DIDMOAD, (Pilz et al 1994). This has been considered to be autosomal recessive (Fraser et al, 1977) but Bunday et al (1992) suggested that DIDMOAD was a mitochondially inherited disorder. The G->A at np 11778 associated with LHON has now been idenfied in a patient with DIDMOAD (Pilz et al, 1994). This raises the possibility that SH has a form of DIDMOAD. However, he has no history of diabetes and the only other neurological symptom was an unexplained episode of paralysis when he was 15.

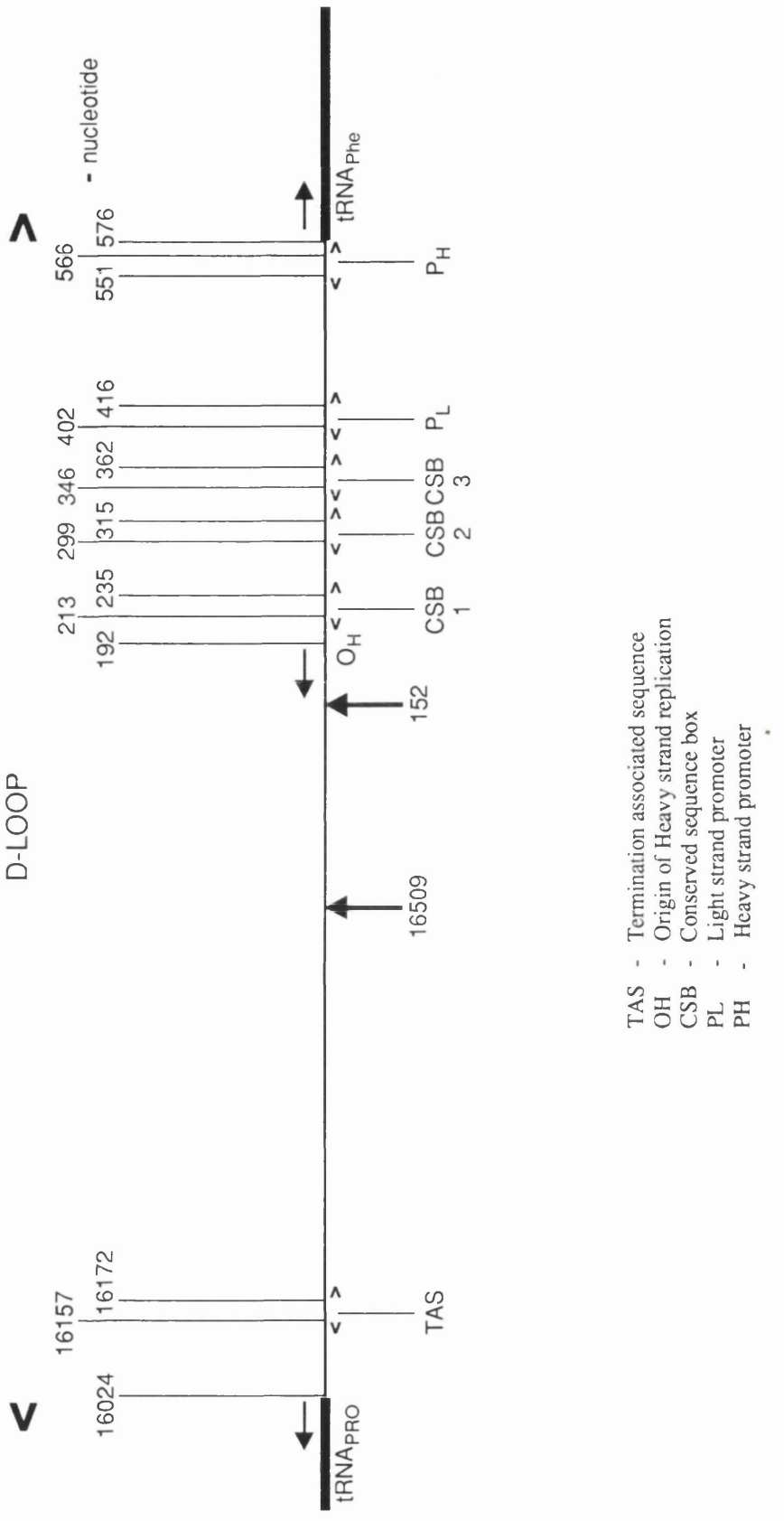


Figure 5:5 Physical map of the human mitochondrial D-loop region indicating the position of the two mutations found in patient SH.

The deafness in SH may be the result of an interaction of the mutation at np 11778 with a nuclear genetic factor, or some environmental factor. If sensorineural deafness is a neurological symptom which can be associated with the 11778 mutation some individuals with the mutation may develop deafness but not optic atrophy. Screening of 33 unrelated patients with sensorineural deafness of no known aetiology for the 11778 mutation indicated that all were wild type at this site (described in detail in Chapter 3).

In view of the paternal relatives of SH with sensorineural hearing loss it cannot be excluded that his deafness has arisen as a result either of genetic factors other than mitochondrial or from some external non-genetic factor.

5:6:3 Interactions of external factors with mitochondrial DNA which may result in the clinical pathology of LHON

Although LHON has been extensively studied over the six years since Wallace identified the np 11778 mutation, the molecular mechanisms which result in disease are not defined. There are wide variations of clinical phenotype even within members of the same family who have the same mutation (Newman, 1993). In addition to optic atrophy as a result of optic nerve damage there are many other manifestations of disease associated with some of the recognised mutations. The 11778 mutation has been found in patients with cardiac conduction abnormalities (Nikoskelainen et al, 1985) and women with a multiple sclerosis-type disease (Harding et al, 1992).

In comparison, how the same clinical phenotype of LHON can result from the variety of mutations reported in different complexes of the oxidative phosphorylation system is unclear. It is likely that the phenotype is not the result of a specific enzyme defect but of a general reduction in mitochondrial energy production which must be very tissue specific. The CNS is the most reliant of all tissues on mitochondrial ATP production but this does not explain the specific effect on the optic nerve.

Attempts have been made to look for alterations in Complex I activity in LHON patients (Howell et al, 1990; Howell et al 1991) and decreased activity found. The relevance of such decreases, found in platelets or tissue culture cells, to their involvement in development of disease in neural tissue is questionable.

Some "external" factor must be implicated in the development of LHON; many theories have been suggested but none proved. As explained in Chapter 1, cigarette smoking was at one time thought to be involved in the development of

LHON symptoms (Wilson, 1965). This idea has never been thoroughly investigated.

The preponderance of males developing LHON led to the suggestion of X-linkage and for a period it was thought that there was linkage to a specific region of the X-chromosome (Vilkki et al, 1991). Re-evaluation of data by Juvonen et al in 1993 and a report by Carvalho et al (1992) resulted in the overall conclusion that there is no X-linkage locus and that the sex bias reflects biological differences between the sexes.

The existence of tissue-specific isoforms in nuclear gene products whose interactions with a mitochondrial gene product give rise to clinical symptoms is a popular theory. Sudoya et al (1992) produced the diagram shown in Figure 5:6 to explain how a changed mitochondrially encoded subunit could be unable to interact with a tissue-specific isoform of a nuclear encoded subunit. What this nuclear gene product could be in LHON remains to be elucidated.

It has been speculated that visual failure in LHON is due to an abnormal immune response against the optic nerve (Harding et al, 1992). It is not clear how mitochondrially encoded polypeptides play a role in the immune response but the mutation at np 11778 causes an Arginine to be replaced by a Histidine both of which are basic, hydrophilic amino acids, and as such could be part of an antigenic determinant. Howell et al (1991) suggested that the change in ND1 brought about by np 3460 mutation could act in a similar manner. It is worth noting that Pallini et al (1988) reported that many LHON patients have increased levels of immunoglobulins in their CSF.

5:6:4 Final comments on future work

It is evident that there is a great deal still to be investigated in the disease known as LHON. The increasing use of automated DNA sequencing will perhaps lead to more LHON patients having their entire mitochondrial genome sequenced so that the relevance of the changes found in this study, in SH, to the development of disease may be established. Additional investigations of deaf patients with respect to the mutations associated with LHON will also clarify whether the two clinical phenotypes are related in SH.

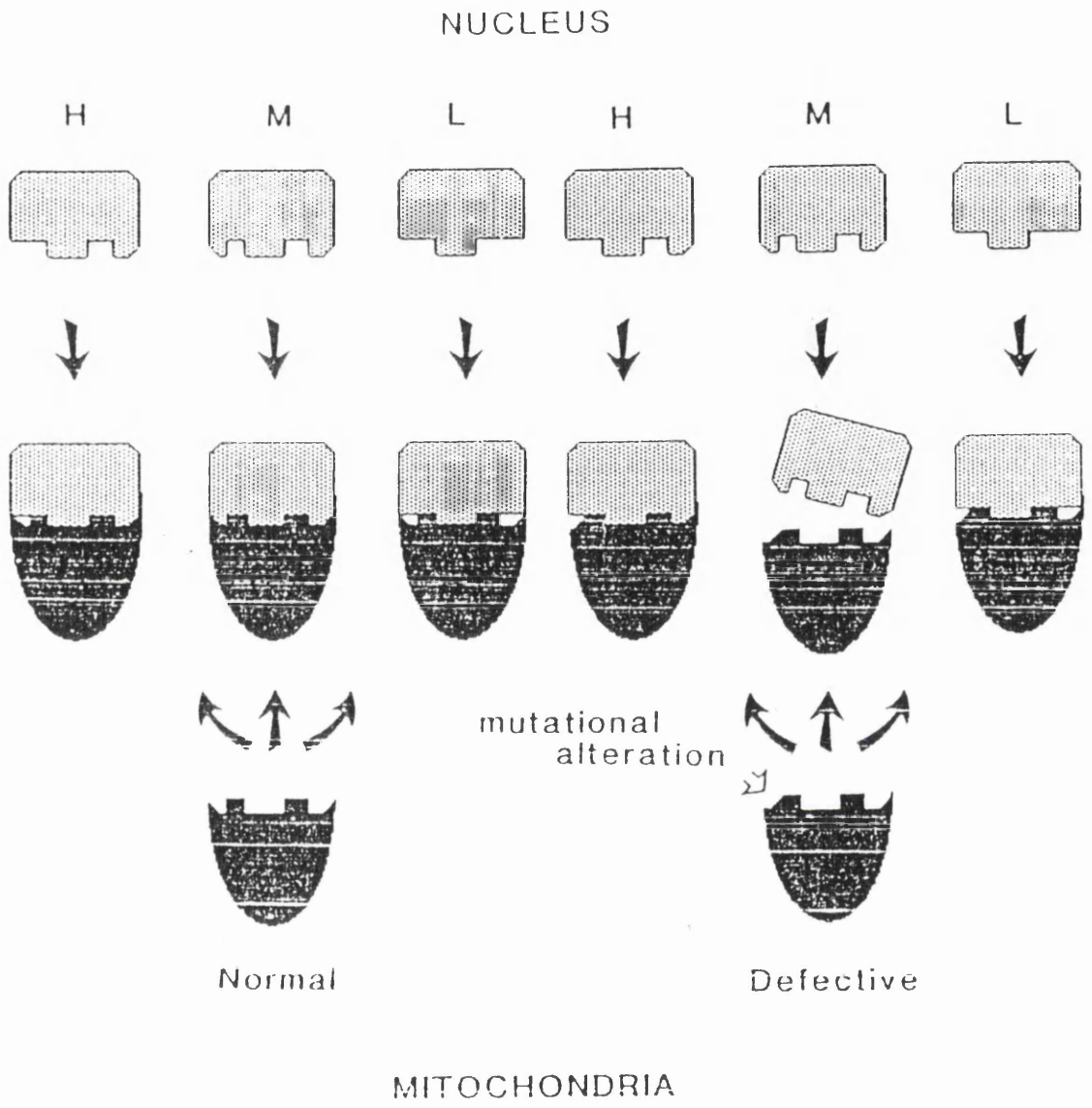


Figure 5:6 Tissue specific expression of mtDNA mutations: nuclear-coded protein subunits of the respiratory enzyme complex as a phenotypic modifier.

Black subunits - mitochondrially encoded
Shaded subunits - nuclear encoded

CHAPTER 6 BIOCHEMICAL INVESTIGATION OF A PERMANENT CELL-LINE CREATED FROM A MEMBER OF THE SENSORINEURAL DEAFNESS PEDIGREE

6:1 CREATION OF PATIENT-DERIVED CELL-LINES

A permanent cell-line was created by EBV-transformation of lymphocytes from patient III-29 (Figure 4:1) in the pedigree described in Chapter 4. Blood was taken from the patient by Guy Vernham and sent to the European Collection of Animal Cell Cultures (ECACC), Porton Down, where the cell-line was created. Cells were supplied to us as growing suspension cultures; aliquots were retained, frozen in liquid N_2, by ECACC. This cell-line was designated as CT001 by ECACC. A control cell-line was provided by Dr Mair Crouch of Yorkhill Hospital, Glasgow: this had been prepared by EBV-transformation of lymphocytes from a female of undefined age suffering from no systemic disease. This cell-line was designated 01MC by our laboratory.

Both cell-lines were maintained as suspension cultures in RPMI with supplements as described in Chapter 2:13. Both grew as typical lymphoblastoid cell-lines: the cells grew in aggregates which were large enough to be seen with the naked eye.

When both cell-lines were established DNA was prepared from each (see Chapter 2:4) and investigated for the np 7445 mutation by means of *Xba*I restriction digestion of PCR products, as described in Chapter 4. As Figure 6:1 shows the patient cell-line, CT001, was found to have lost the site at np 7440, whereas the normal control cell-line, 01MC, was wild-type. It may be infered that CT001 had retained the A->G mutation at np 7445. This procedure was undertaken in order to be sure firstly that the patient cell-line had maintained the mutation in culture, and secondly that the normal control did not by chance have the np 7445 mutation.

6:2 MITOCHONDRIAL METABOLISM IN PATIENT AND CONTROL CELL-LINES

A number of ways of measuring mitochondrial metabolism in cultured cells are available. Those investigated here were determination of the ratios of lactate to pyruvate produced by cells in culture; complex II/III activities, complex IV activity and growth in selective media.

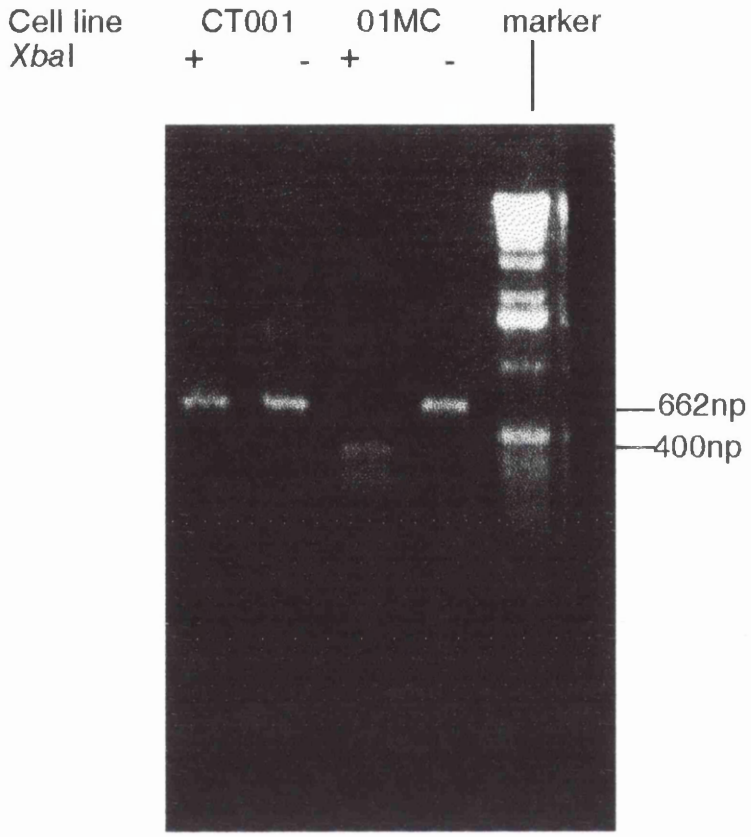


Figure 6:1 Digestion with XbaI of PCR products amplified using template DNA prepared from the cell-lines CT001 and 01MC and forward primer FR31/7178B, reverse primer FR32/7840L.

6:2:1 Measurement of lactate/pyruvate ratios in patient and control cell-lines

The measurement of lactate/pyruvate ratios is a general method of estimating levels of oxidative phosphorylation in cell-lines or tissue samples (Robinson et al, 1987). Lactate is the product of anaerobic respiration; when oxidative phosphorylation is not functioning properly and cells or tissues produce ATP via glycolysis, lactate levels rise. Lactate accumulation in body fluids (e.g. blood, CSF) is considered a useful marker of mitochondrial disease (Tulinius et al, 1991). Lactate/pyruvate ratios were measured to estimate levels of oxidative phosphorylation in cells with the np 7445 mutation, CT001, and normal control cells without this mutation, 01MC. This was done by incubating cells as described in Chapter 2:19 and subsequently measuring the lactate and pyruvate produced. The enzymatic assay measures the levels of NADH by the optical density (OD) at 340 nm. The reaction is:

lactate + NAD $\rightleftharpoons$ pyruvate + NADH

and the enzyme which acts is lactic dehydrogenase. Standard curves for both lactate and pyruvate are produced by carrying out the assay on a range of concentrations of the two substrates (see Chapter 2:19). From these, OD_{340} readings obtained from cell-lines after 48 hours incubation could be converted to concentrations and expressed per mg of protein.

Results are given in Table 6:1. The levels of lactate produced were extremely low in all experiments. Lactate/pyruvate ratios were calculated from the results of three such experiments in which cells were incubated for 48 hours prior to the assay; the ratio for 01MC was 18.2 ± 5.5, that for CT00I was 15.9 ± 4.8. These results are within the limits of experimental variation and indicate no significant difference in oxidative phosphorylation between the two-cell-lines.

6:2:2 Complex II + III activity in the two cell-lines

Complex II + III activity was measured in disrupted cells for both 01MC and CT001 cell-lines using the succinate/cytochrome *c* reductase (SCCR) assay (see Chapter 2:18). The results of one experiment are shown in Figure 6:2 ; the assay was carried out in duplicate on one batch of cells, OD_{550} readings (absorbance of cytochrome c) were taken and a mean calculated. The mean change in OD_{550} values was 0.005/min for both cell-lines. When the respective protein concentrations were taken into account this gives figures of 0.73 nmol substrate/min/mg protein for cell-line CT001 and 0.93 nmol substrate/min/mg protein for cell-line 01MC. These figures are extremely low when compared to those quoted by Shoffner et al (1992) but this reflects the fact that experiments

CELL-LINE	LACTATE	PYRUVATE	LACTATE/ PYRUVATE
	1.2 mM	57.4 μM	20.9
01MC	521 μM	44.3 μM	11.8
	1.4 mM	64.4 μM	21.6
			Mean ± SE
			18.1 ± 5.5
	986 μM	65 μM	15.2
CT001	774 μM	67 μM	11.4
	1.4 mM	68 μM	20.9
			Mean ± SE
			15.8 ± 4.8

Table 6:1 Lactate and pyruvate production ratios, after 48 hours, in three separate experiments, calculated from OD_{340} values obtained in the enzyme assays described in Chapter 2:19. Concentrations of lactate and pyruvate are per mg of protein.

Pyruvate standards were 1 μM - 200 μM; lactate standards were 10 μM - 20 mM

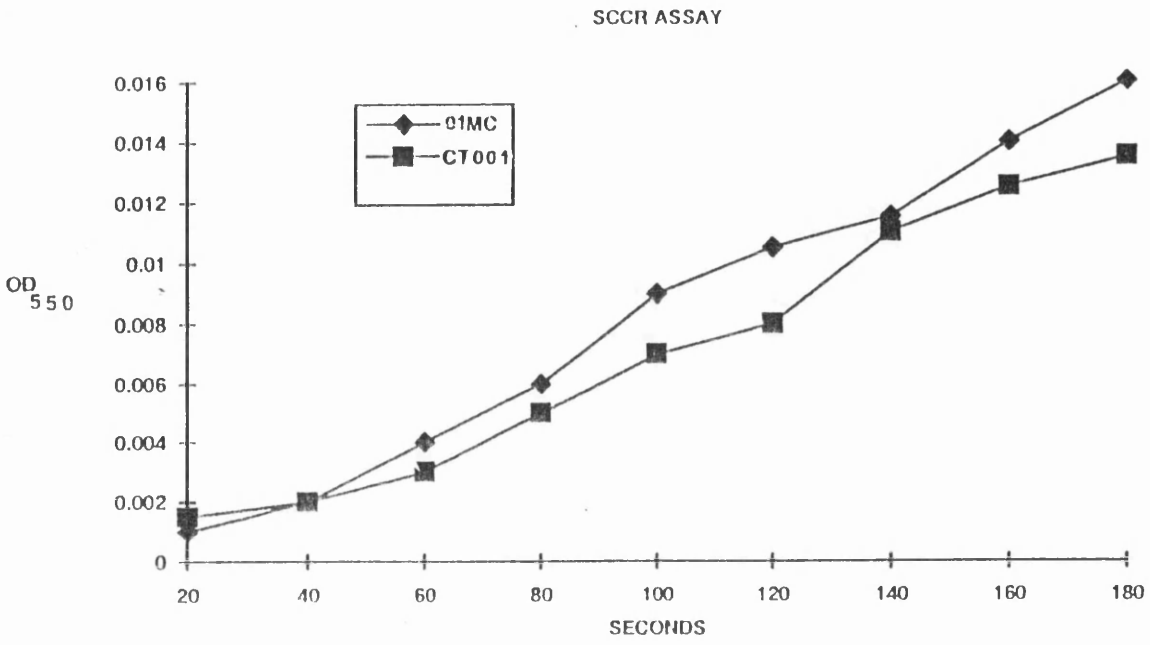


Figure 6:2 Succinate/cytochrome-*c* reductase (SCCR) assay measuring complex II + III activity in the patient-derived cell-line, CT001, and the control cell-line 01MC. This experiment was carried out in duplicate on 1.25 x 10^5 cells which had been disrupted by freezing and thawing

were done using whole cells, while those of others used purified mitochondria and their figures are per mg of mitochondrial protein.

6:2:3 Complex IV activity (Cytochrome-*c* Oxidase assay)

The cytochrome-*c*-oxidase (COX) assay measures the oxidation of cytochrome-*c* by complex IV of the oxidative phosphorylation system in mitochondria. This activity was measured as absorbance at OD_{550} in both 01MC and CT001 cell-lines: results shown in Figure 6:3 are the mean of duplicate readings. Calculated from these results the mean change in OD_{550} for 01MC was 0.038/min and for CT001 was 0.034/min. These results convert to 4.3 nmol/min/mg protein for CT001 and 4.8 nmol/min/mg protein for 01MC.

The results of the SCCR and COX assays can be expressed as a ratio to give a way of measuring complex IV activity relative to the flux through complexes II + III (I. J. Holt, personal communication). In most cases of mitochondrial disease complex IV is the most severely impaired of the respiratory chain activities. Expressed in this way the COX/SCCR ratio for 01MC was 5.1 and for CT001 5.8.

6:3 MEASUREMENT OF GROWTH OF THE TWO LYMPHOBLASTOID CELL-LINES BY MTT ASSAYS

The number of viable cells in a culture can be measured by Mosmann's MTT assay (Mosmann, 1983). The ability of the two cell-lines to survive under various culture conditions was investigated and compared using a modification of this assay as described in Chapter 2:20

6:3:1 Growth of cell-lines on galactose

The ability of the two cell-lines to grow using Galactose as carbon source was measured, by the MTT assay, in cells maintained in RPMI containing galactose substituted for glucose. In Figure 6:4 the growth curves of both cell-lines are shown under both conditions: in RPMI containing glucose (Figure 6:4:A) and in RPMI containing galactose (Figure 6:4:B). The graphs show that the patient derived cell-line, CT001, grew better than the control, 01MC, under both conditions. Mean doubling time was calculated by plotting $\log_{10}$ of the OD_{570} readings against time (not shown). The length of time taken for the OD readings to double can be estimated from the shape of this graph. In glucose the doubling time for the CT001 cell-lines was 32 hours, and 55 hours for the 01MC cell-line; in galactose the figures were 56 hours and 80 hours respectively. Calculated from these figures there was an increase in the mean doubling time in Galactose of 1.4

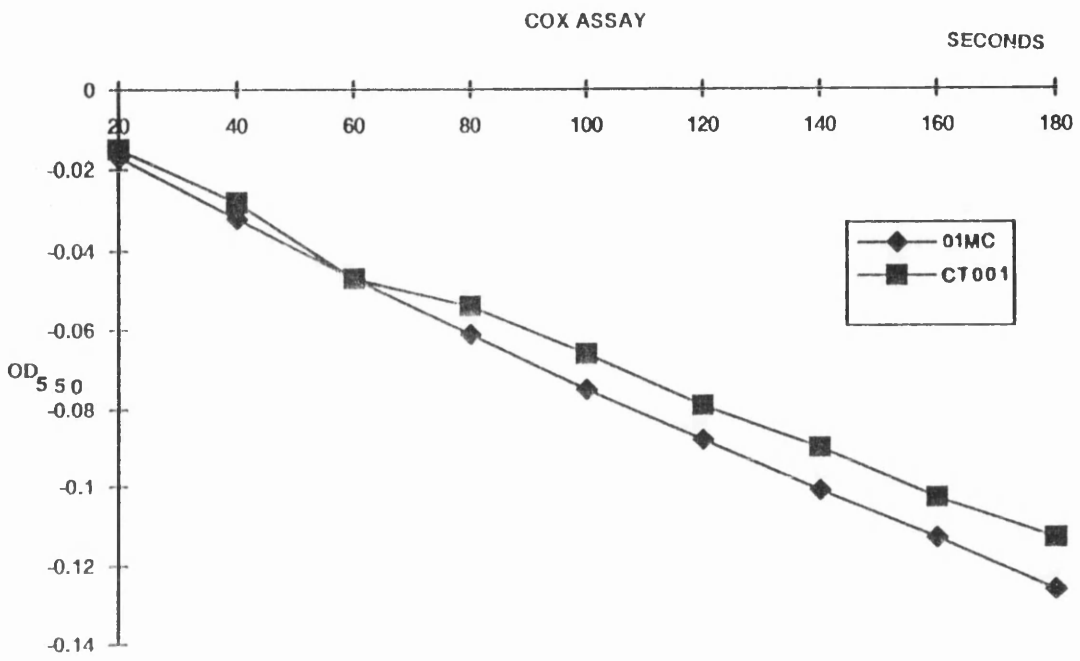


Figure 6:3 Cytochrome-*c* oxidase assay measuring complex IV activity in the cell-lines CT001 and 01MC. The assay was carried out in duplicate on one batch of cells; 1.25 x 10^5 cells were disrupted by freezing and thawing and treatment with 10% digitonin.

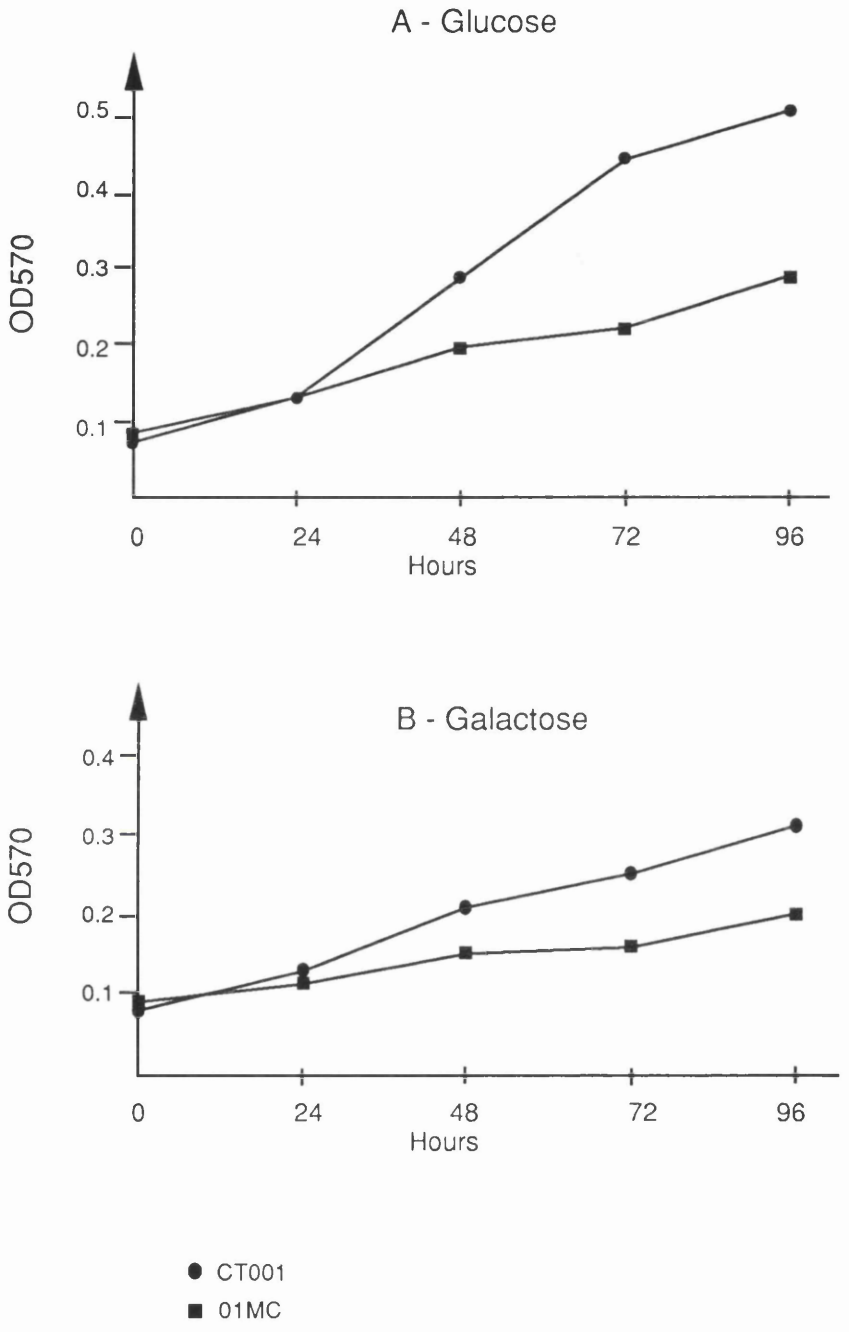


Figure 6:4 Measurement of cell growth in various media by MTT assays.

A: growth in RPMI containing glucose
B: growth in RPMI containing galactose

The data plotted are the means of two experiments in which measurements were taken in triplicate. Standard errors (not shown) were all less than 0.05 OD units.

times for 01MC and 1.7 times for CT001. These ratios are probably not significantly different, given the experimental errors inherent in the technique.

6:3:2 Growth of patient and control cell-lines in the presence and absence of streptomycin

Standard growth conditions for lymphoblastoid cell-lines include 100 μg/ml of streptomycin and 100 units/ml of penicillin in the RPMI growth medium, in order to combat bacterial contamination of cell cultures. In an attempt to gauge whether inclusion of higher doses of streptomycin in the culture medium had any effect, either inhibitory or enhancing, on the growth of the two cell-lines. MTT assays were carried out on cells grown under conditions of no streptomycin, 200 μg/ml streptomycin and 400 μg/ml streptomycin, all with 100 U/ml penicillin. Figure 6:5 shows the growth curves in each situation: Figure 6:5:B indicates that both cell-lines can grow as well without streptomycin as they can with 100 μg/ml. Figure 6:5:C and D show the results of two experiments in which growth was measured in 200 μg/ml and 400 μg/ml streptomycin. The two sets of results are shown separately due to the differences in the results. Comparison of growth curves shown in Figure 6:5:A (control 100 μg/ml streptomycin) and C indicates that in the first experiment (19.8.94) conditions of 200 μg/ml streptomycin resulted in a marked decrease in growth of both cell-lines. Comparison of Figure 6:5:A and D indicates that 400 μg/ml streptomycin caused stasis of the control cell-line, 01MC, whilst growth of CT001 was decreased but not stopped. Contrary to these results, in the second experiment (16.9.94) at a concentration of 200 μg/ml there was no decrease in the growth of CT001 and only a small decrease in that of 01MC. But at 400 μg/ml there was a decrease in the growth of both cell-lines, although a smaller effect was seen in the patient derived cell-line. These results require clarification by repetition of the experiment.

6:3:3 Growth of cell-lines in the presence of chloramphenicol

Chloramphenicol inhibits mammalian mitochondrial protein synthesis (Lamb et al, 1968). If there is an impairment of mitochondrial protein synthesis in the cell-line CT001 with the np 7445 mutation, which is not sufficient to cause a decrease in respiration under normal culture conditions, stressing the cells by growing them in the presence of chloramphenicol may reveal a growth defect in the CT001 cells.

Some mutations in the 16S rRNA gene of mitochondrial DNA are known to confer resistance to chloramphenicol (Howell & Lee, 1989). None of the

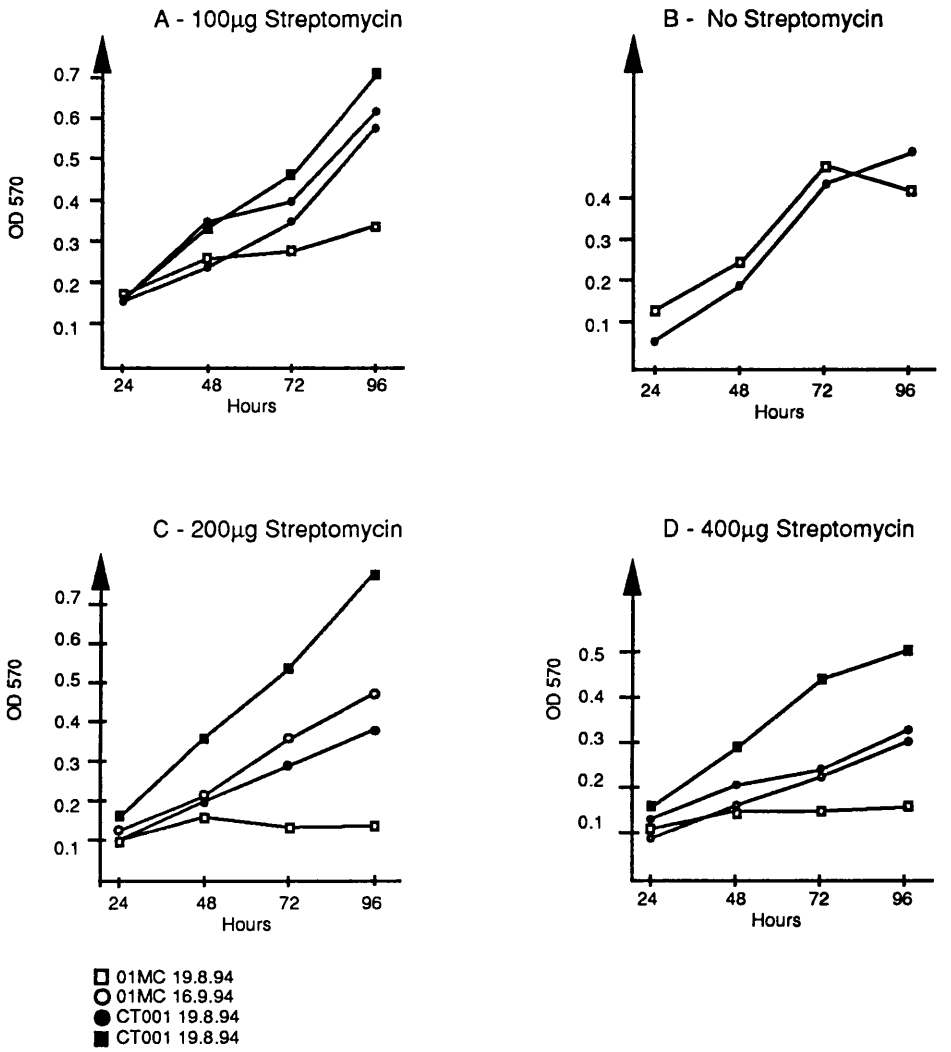


Figure 6:5 Growth curves (by MTT assay) of cell-lines 01MC and CT001 in the presence of: 100µg/ml streptomycin (A), no streptomycin (B), 200µg/ml streptomycin (C), and 400µg/ml streptomycin (D). Penicillin (100 U/ml) was present in the RPMI culture medium in all experiments. Each growth curve is the mean of triplicate samples. Standard errors (not shown) were all less than 0.05 OD units.

mutations of mitochondrial DNA found in patient III-29, from whom the CT001 cell-line was created, are known to be chloramphenicol resistance mutations and it was decided to test both cell lines for their ability to grow in RPMI containing chloramphenicol. A concentration of 1 mM was found to kill (an OD_{570} reading of approximately zero in MTT assays) both cell-lines within 48 hours. The growth curves in 0.1 mM chloramphenicol are shown in Figure 6:6, although growth under control conditions (Figure 6:6:A) was poor in this experiment, it can be seen that growth in the presence of 0.1 mM chloramphenicol was similarly reduced in the two cell-lines. Constraints of time did not permit the experiment to be repeated in order to confirm this result.

6:4 PRELIMINARY ANALYSIS OF MITOCHONDRIAL TRANSCRIPTS IN THE PATIENT-DERIVED AND CONTROL CELL-LINES

The position of the np 7445 mutation at the boundary of the COI gene on one strand and the $tRNA_{ser(UCN)}$ gene on the other could result in a difference in RNA processing (as outlined in Chapter 4:8:4). Preliminary experiments were undertaken to investigate whether there was a difference in the length or level of the COI transcript, between the patient-derived and control cell-lines.

RNA was prepared from whole tissue-culture cells as described in Chapter 2:14 . After electrophoresis through a formaldehyde agarose gel and transfer to postively charged nylon membrane, the RNA was hybridised with DIG-random labelled PCR product. This product had been amplified from template DNA prepared from a blood sample donated by a normal control, forward primer FR29/6456B and reverse primer FR30/7252L. These primers amplify a 790 np product which contains only sequence within the COI gene. Thus when used to hybridise to a Northern blot which is subsequently washed a high stringency, only the COI transcript should hybridise.

Figure 6:7 shows an autoradiograph of a Northern blot hybridised in this way. The apparent difference in signal between the two RNA samples was due to a loading difference as estimated from examination of the methylene blue stained filter prior to hybridisation.

In order to confirm and extend these results Dr Howy Jacobs carried out a Northern analysis of the same RNA samples using a high specific activity probe made by ^{32}P-labelling of the same PCR product as used in the experiment described above. Several exposures of the autoradiograph produced are shown in Figure 6:8; showing the same major transcript as was seen with the DIG-labelled

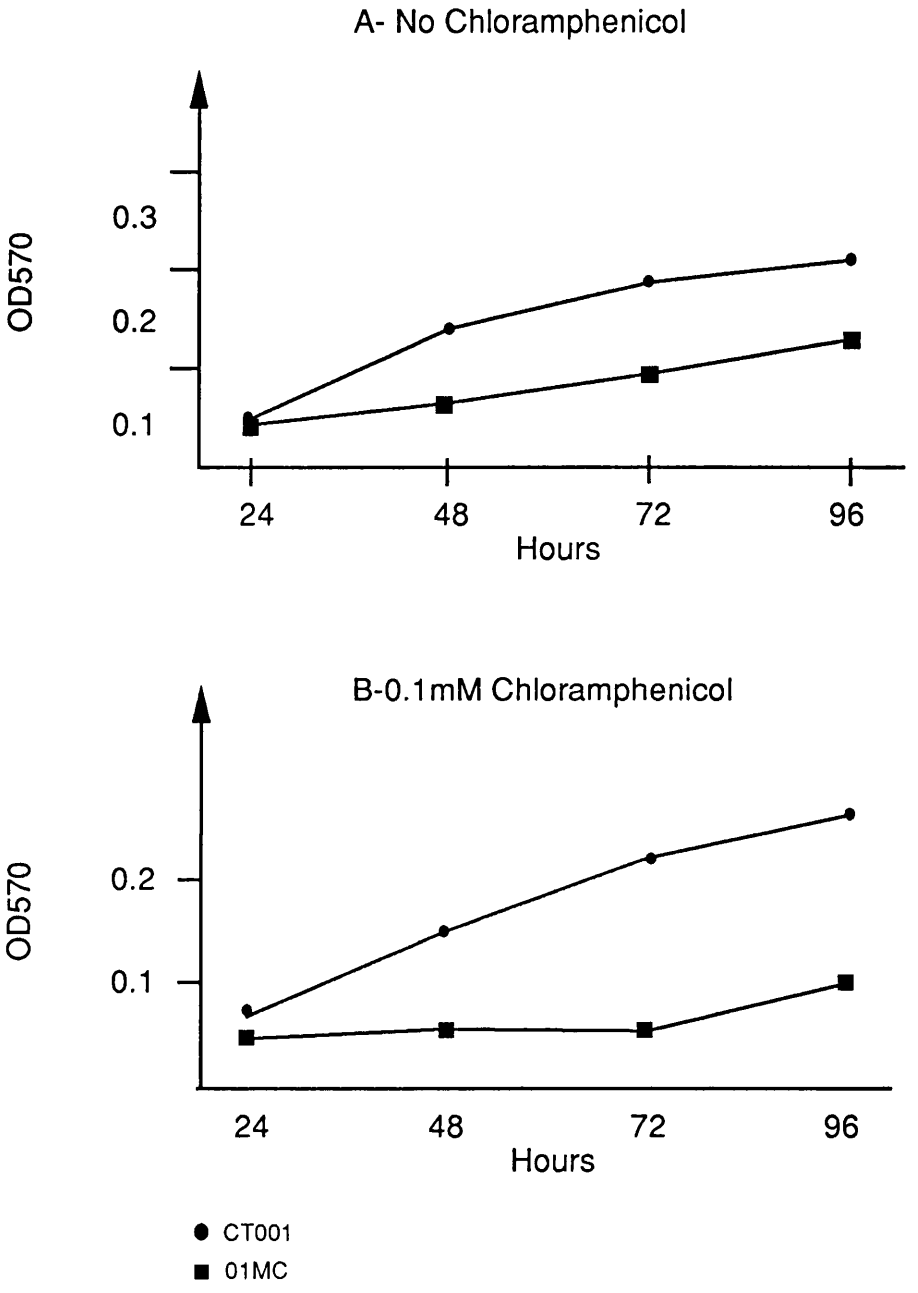


Figure 6:6 Growth curves (by MTT assay) of cell-lines 01MC and CT001 on RMPI without chloramphenicol (A) and with 0.1 mM chloramphenicol (B). Results are the mean of triplicate samples from one experiment, standard errors were all less than 0.05 OD units (not shown).

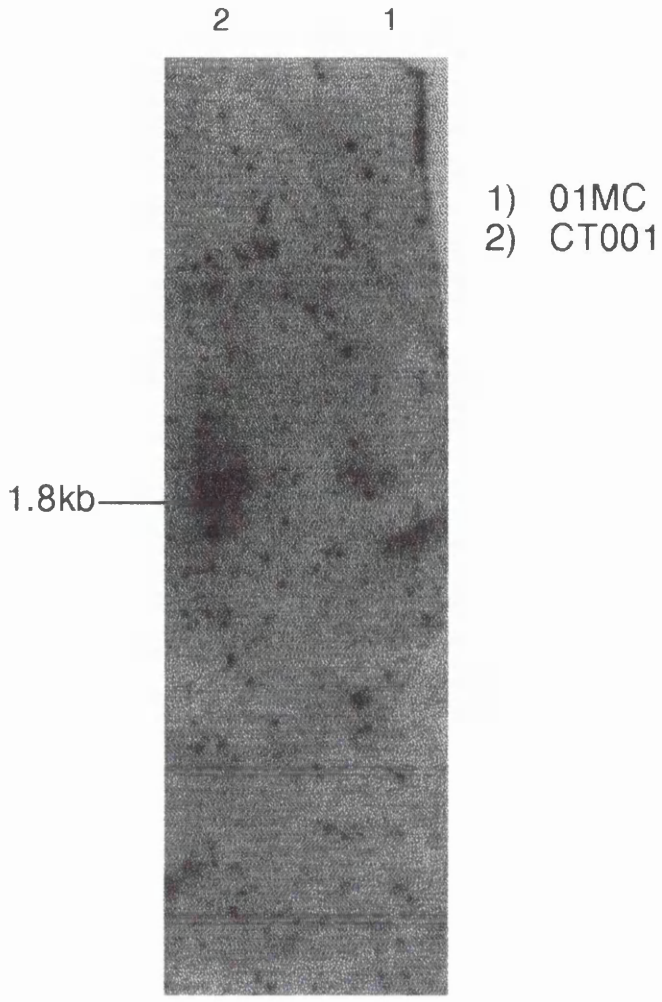
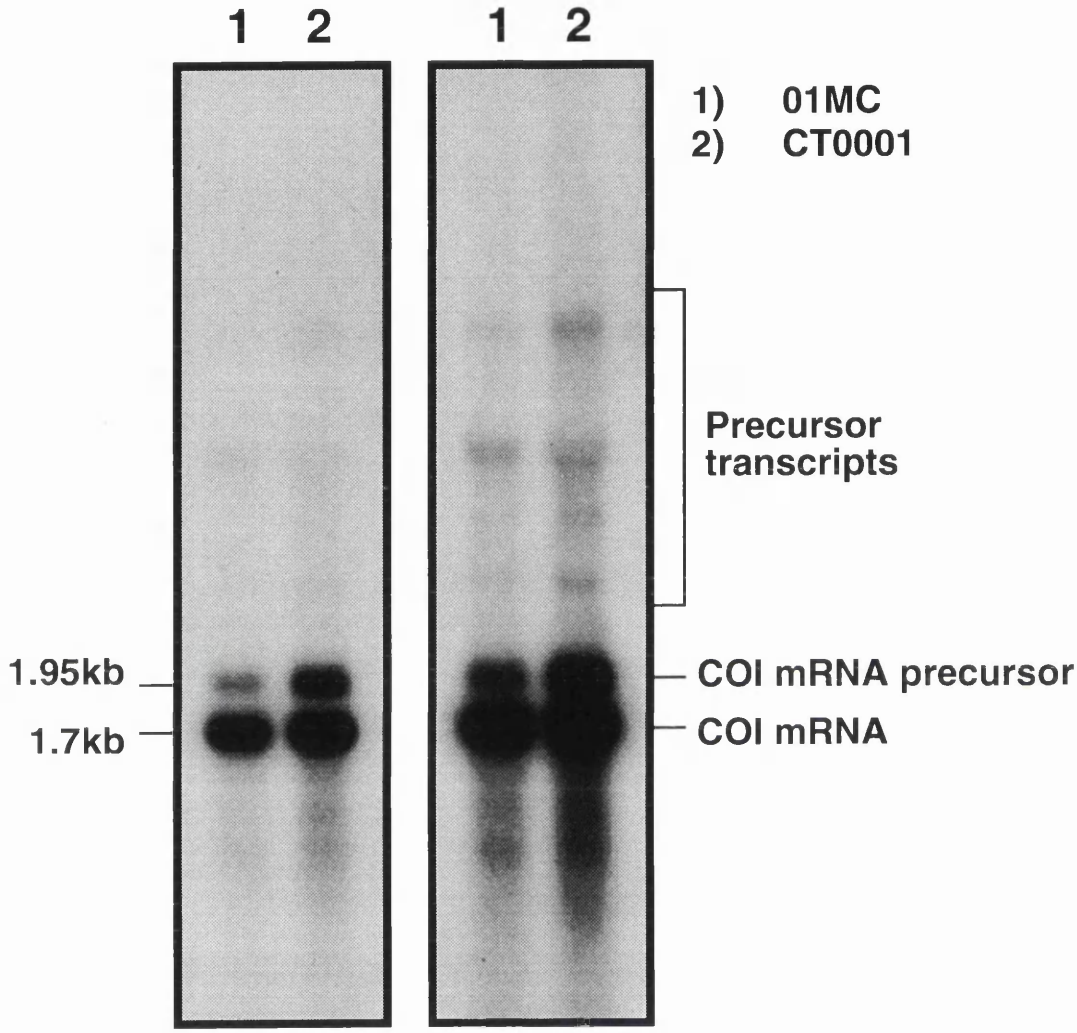


Figure 6:7 Northern blot of 10µg of total RNA extracted from CT001 cells and 01MC cells, hybridised with DIG-random labelled PCR product created using primers FR29/6456B and FR30/7252L. Bands of the same size corresponding to the COI transcript (approximately 1800 nucleotides) are seen in both RNA samples.

Figure 6:8 Northern blot of 10μg of total RNA, extracted from CT001 cells and 01MC cells, hybridised with ^{32}P-dCTP random labelled PCR product created using primers FR29/6456B and FR30/7252L and normal human control DNA as template. Hybridisation was carried out in 50% formamide, 5XSSC at 43°C; the final wash was in 0.1 XSSC/0.1% SDS at 55°C. This Northern analysis was carried out by Dr Howy Jacobs on RNA samples prepared by the author.


1 2
1 2
1) 01MC
2) CT0001
Precursor transcripts
1.95kb
1.7kb
COI mRNA precursor
COI mRNA

probe; in addition a slightly larger transcript was seen in both RNA samples. This is probably the longer precurser of COI which also contains sequence for the 5 antisense tRNAs upstream of the COI gene. Neither the DIG-labelled probe nor the ^{32}P-labelled probe detected any difference in the length of the COI transcript between the RNA from patient-derived cells and control cells, nor any difference in the structure of precursor-like transcripts containing COI sequences. When adjusted for relative loading (Figure 6:8 plus additional data obtained by Dr Jacobs, not shown) the level of COI mRNA in the two cell-lines is very similar.

6:5 MEASUREMENT OF MITOCHONDRIALLY-ENCODED PROTEIN SYNTHESIS IN CELL-LINES

The synthesis of mitochondrial-encoded proteins was investigated by ^{35}S-methionine incorporation in the presence of emetine, which is an inhibitor of cytosolic protein synthesis. ^{35}S-labelled proteins were prepared as described in Chapter 2:16 and electrophoresed through a 12% polyacrylamide-SDS gel. The proteins were visualised by coomassie blue staining of the gel before fixing and treatment with "Amplify". The radio-labelled proteins were detected by fluorography. Figure 6:9 is one such experiment in which cells were preincubated in methionine-free medium in the presence of emetine. As shown the bands corresponding with COI, ND5, ND4, Cyt *b*, COIII, ATPase 6, ND6 and ND3 have been tentatively assigned in the 10^{-4} M emetine tracks. In this experiment chloramphenicol was not included; another experiment in which chloramphenicol was present in addition to 10^{-4} M emetine, in one set of samples, showed that the prominent assigned bands shown in Figure 6:9 were not present when chloramphenicol was included (data not shown). This indicates that the proteins seen in the 10^{-4} M track are translated in the mitochondria. The most important thing to note is that there are no additional bands, no loss of bands and no difference in the intensity of the bands in the CT001 samples.

6:6 DISCUSSION

In order to investigate the biochemical and molecular effects of the mutation at np 7445, a permanent cell-line was created from lymphocytes of the index case. As a control a similar lymphoblastoid cell-line was studied, derived from normal control lymphocytes.

6:6:1 Biochemical investigations of patient-derived and normal cell-lines

Many of the studies of mitochondrial diseases have measured, directly or indirectly, the oxidative phosphorylation activity of cells from patients in

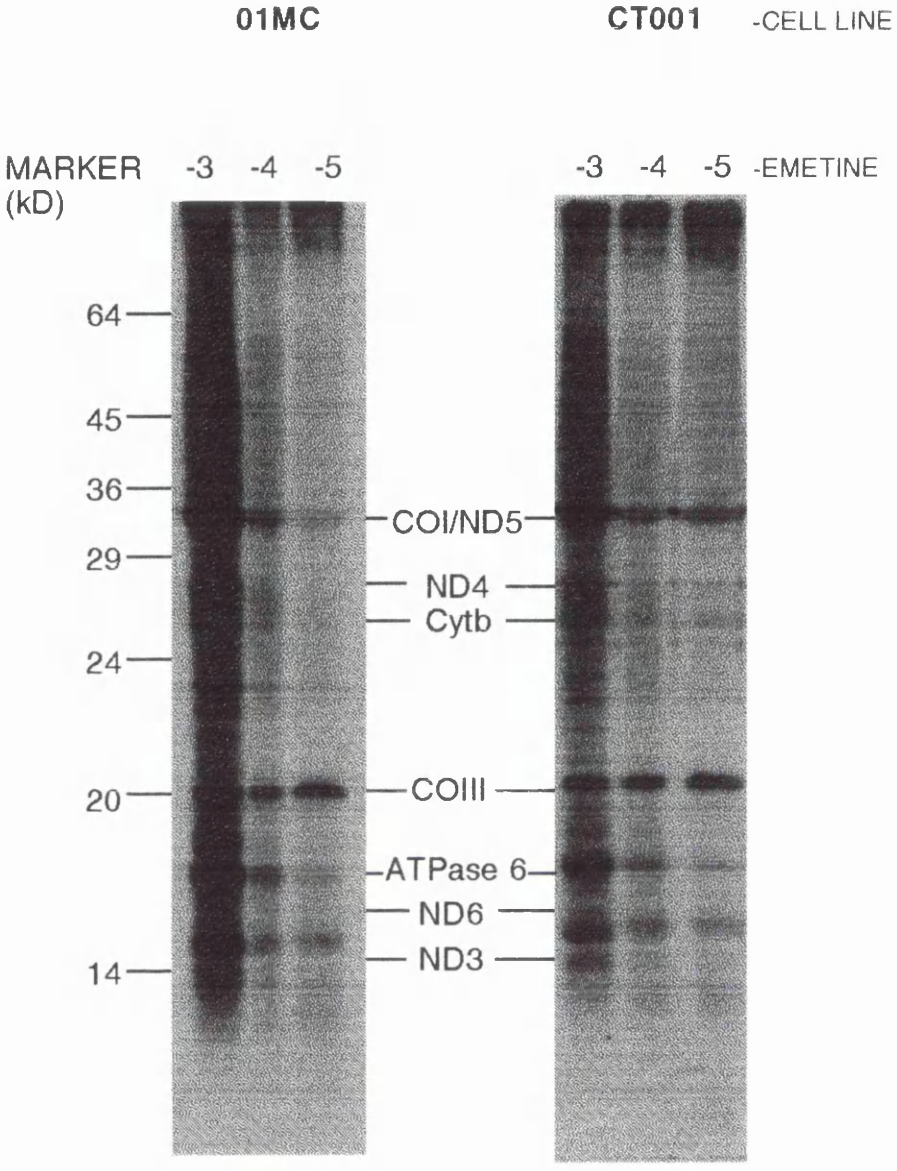


Figure 6:9 Fluorograph of ^{35}S-Methioninelabelled proteins electrophoresed through SDS-polyacrylamide. 1 x 10^6 cells were labelled with 25 µCi of ^{35}S-Methionine at each concentration of emetine ie 10^{-3}, 10^{-4} and 10^{-5}. Proteins were assigned according to sizes given by Bourgernon et al (1992).

comparison with those from normal controls. In this study lactate and pyruvate production were measured and a lactate/pyruvate ratio calculated. The ratios of 18.1 for the normal cell-line, 01MC, and 15.8 for the patient-derived cell-line, CT001 are not significantly different although slightly lower than the ratios reported by Robinson et al (1987) for control fibroblast cell-lines (ratios 21.5 to 26.9). Of most importance in attempting to identify a biochemical phenotype attributable to the mitochondrial genotype of the patient-derived cell-line is that there is no difference in the lactate/pyruvate ratios. This suggests no difference in oxidative phosphorylation.

The activity of individual complexes was measured by the SCCR and COX assays; results are shown in Figure 6:2 and Figure 6:3. It is clear that there is no difference in Complex II + III activity, measured by SCCR, or Complex IV activity, measured by COX, between the two cell-lines. If the results of these two assays are expressed as a ratio the figures are COX:SCCR of 5.1 for 01MC and 5.8 for CT001. A review of literature indicates that ratios in normal human controls range from 2.2 (Wallace et al, 1988) to 7.5 (Haginoya et al, 1993). In a number of mitochondrial myopathies muscle biopsies were investigated by these assays and gave equally variable results: 2.4 in mitochondrial encephalomyopathy (Servidei et al, 1987), 2.4 and 3.7 in KSS (Shoffner et al, 1989), 3.9-6.9 in Leigh's disease (Shoffner et al, 1992).

The similarity of the ratios obtained from the two cell-lines is in agreement with the lactate/pyruvate results. Both indicated that the mutation at np 7445 in the patient-derived cell-line is not resulting in a decrease in oxidative phosphorylation, at least in lymphocytes. A decrease in oxidative phosphorylation in lymphocytes would presumably result in much more generalised systemic disease than is found in the pedigree under study. There are no clinical symptoms apart from sensorineural deafness in the majority of the family (the exceptions being III-10 and III-15 who have ophthalmological symptoms). Lymphoblastoid cells are not the ideal choice of cells to use in an investigation of deafness in this family. Neuronal tissue or tissue derived from the cochlea would give much more meaningful results. Samples of these tissues are, however, difficult to obtain and maintain. It would also be hard to justify such studies ethically.

6:6:2 Growth of patient and control cell-lines under various conditions

The ability of cells to grow in various culture conditions was investigated using a modified MTT assay. MTT is a tetrazolium salt, 3-(4, 5-dimethyl-thiazol-2-yl)-2.5-di-phenyltetrazolium bromide which is bright yellow in colour and produces a

dark blue formazan product when incubated with live cells (Mosmann, 1983). A homogeneous solution which can be measured for optical density is produced by addition of 10% SDS (Tada et al, 1986). The tetrazolium ring of MTT is cleaved in active mitochondria by dehydrogenases.

This method was used to measure growth of the two cell-lines in RPMI (with additives as shown in Chapter 2) with galactose as carbon source, in the presence of chloramphenicol and in various concentrations of streptomycin. In general the patient-derived CT001 cells grew better than the control 01MC cells under all conditions tested.

Neither cell-line grew as well on galactose as on glucose (see Figure 6:4), but the increase in doubling time was similar for both cell-lines indicating no difference in their ability to utilise galactose as a carbon source. Galactose is converted to galactose-1-phosphate, a reaction which requires ATP, and this is converted to UDP-galactose. The UDP-glucose formed is then catabolised in the same way as that formed from glucose. However, the rate of galactose utilisation via this pathway is held to limit the rate of supply of precursors for glycolysis such that the cell is unable to obtain sufficient ATP from glycolysis alone for growth and/or survival. (Sun et al, 1975; Lehninger, 1982). This assay is therefore a further test for respiratory impairment.

At a concentration of 1 mM, chloramphenicol was found to kill both cell-lines, but at the lower concentration of 0.1 mM growth rates for both cell-lines were reduced by approximately the same factor (see Figure 6:6). The results of this preliminary experiment indicate that the cell-lines did not respond differently to growth under conditions of stress (i.e. chloramphenicol). The mutation at np 7445 does not appear to be resulting in a decrease in growth in this situation.

Sensitivity to higher than normal levels of streptomycin in growth medium was measured by MTT assays. Figure 6:5 shows that the two experiments in which growth was measured in 200 μg/ml and 400 μg/ml streptomycin gave different results. Unfortunately time did not permit me to repeat these assays, but six control lymphoblastoid cell-lines have been obtained from ECACC to establish the range of sensitivity to streptomycin in control lymphoblastoid cell-lines. Only when these assays have been completed, in triplicate (in addition to re-assay of 01MC and CT001) will any conclusion be possible regarding the effect of the np 7445 mutation on the streptomycin sensitivity of cell-line CT001.

If it is found to be significantly less sensitive to streptomycin than normal then I suggest that fesh blood samples be obtained from patient III-29 and a number of

lymphoblastoid lines created from this patient which could be similarly tested. This would reveal whether any difference in sensitivity observed between the two cell-lines was a real phenotypic effect of the mitochondrial genotype.

As described in Chapter 4 the position of the np 7445 mutation is at the extreme 3' end of the $tRNA_{ser(UCN)}$. This potentially implicates the mutation in mitochondrial protein translation although in the experiments described in Section 6:4, above, no mis-translation of mitochondrial proteins was detected in the cell-line with the mutation, CT001.

Streptomycin has been shown to bind the 16S rRNA in *E. coli* and is proposed to cause mis-translation in mitochondria in a similar manner to its binding to the 16S rRNA of prokaryotes (Prezant et al, 1993). The experiments to investigate the growth of cell-lines CT001 and 01MC in the presence of streptomycin were undertaken to test whether the combination of the np 7445 mutation with the affect of streptomycin would result in a growth difference. If, when the experiments are repeated, a difference is found between the growth CT001 and 01MC then all the experiments described in this Chapter, lactate/pyruvate, SCCR assay, COX assay, mitochondrial protein synthesis should be repeated on cells grown in increased concentrations of streptomycin.

The results of similar MTT assays as performed on 01MC and CT001 on a range of lymphoblastoid cell-lines under various growth conditions would clarify whether the shorter doubling time observed for CT001 under normal growth conditions is within the normal range or is really a result of phenotypic expression of the genotype, nuclear or mitochondrial, of this cell-line.

6:6:3 Investigation of the effect of the np 7445 mutation on mitochondrial RNA

The Northern analysis shown in Figure 6:7 and Figure 6:8 indicated that there is no difference in the size of the COI transcript between the two cell-lines. In Figure 6:8 where the larger precurser transcript is present in addition to the mature COI transcript of approximately 1800 nucleotides, there is no differently sized or additional band present in the patient cell-line. This result suggests that the A->G change at np 7445, which is at the overlapping boundary between the COI gene on the major coding strand and the $tRNAser_{(UCN)}$ gene on the other strand, makes no difference to the length of the COI mRNA. The AGG created is presumed to act as a stop codon in the same way as the wild-type AGA, unlike the G ->A change at np 7444, reported by Brown et al (1992) in Leber's patients,

which they claimed, causes an increase of 3 amino acids in the length of the COI polypeptide.

6:6:4 Investigation of the effect of the np 7445 mutation on mitochondrially encoded protein synthesis

The possible effects of the np 7445 mutation on mitochondrially encoded protein synthesis was investigated by ^{35}S-Methionine incorporation in the presence of emetine. Figure 6:9 shows a Fluorogram of one such experiment. Assignment of bands is difficult as not all mitochondrially-encoded proteins can be identified. However, this is true for both cell-lines, and must be due to experimental procedure. Both cell-lines show the same pattern of bands and each at similar intensities, indicating no difference in the sizes of any of the mitochondrially-encoded proteins or in the levels of their synthesis.

In contrast similar experiments on cybrid cell-lines containing mitochondrial DNA with the MELAS np 3243 mutation showed variable decreases in some of the mitochondrially-encoded polypeptides (King et al, 1992). In particular there was a decrease in the larger polypeptides such as ND2. A correlation between the number of leucine (UUR) codons in the polypeptide genes and the decreases observed was investigated by King et al but not found. It would be imagined that if the np 7445 mutation did decrease the charging efficiency of the $tRNA_{ser(UCN)}$ some effect would be seen, particularly on mitochondrially-encoded polypeptides with large numbers of serine residues specified by UCN codons.

Mis-charging of the $tRNA_{ser}$ with another amino-acid would result in proteins which are altered in their primary and consequently, secondary and tertiary structure. This would ultimately result in a loss of function of mitochondrially encoded proteins. There is no evidence for gross alterations in the structure ofthe mitochondrial encoded proteins of the lymphoblastoid cell-line, CT001 and it must be concluded that mis-charging is not occurring at a substantial level in this cell-type.

6:6:5 Concluding remarks

All the preliminary data presented in this Chapter indicate that the mutation at np 7445 in the pedigree with sensorineural deafness does not result in a decrease in oxidative phosphorylation, at least in lymphocytes. Nor does the mutation result in alteration of the size or amount of COI mRNA, or in the level or size of mitochondrial encoded proteins. However, the clinical phenotype in the pedigree is extremely tissue-specific, limited to the sensorineural auditory system and it is

perhaps not surprising to find no identifiable phenotype in lymphoblastoid-cell cultures.

In Chapter 4 of this thesis the involvement of a nuclear genetic factor was suggested in the development of the sensorineural deafness phenotype exhibited in some members of the pedigree. The nuclear background in which the mitochondrial genotype is expressed may be extremely important in the expression of a measurable biochemical phenotype. Experiments similar to those described by Bodnar et al (1993), may help to resolve the role of the nuclear background. In these experiments enucleated patient fibroblasts were fused with rho° fibroblasts created from normal controls and the resulting cybrids used to investigate the influence of the nuclear genome on the function of the mitochondrial genome. However, until a culture system can be devised in which there is a readily detectable phenotypic difference between cells with the np 7445 mutation and wild-type, it is difficult to envisage exactly how these experiments could be done. As is further discussed in Chapter 7, it is also difficult to suggest other means of investigating the molecular mechanisms of disease in this family which are not extremely invasive and, as such, probably unethical.

CHAPTER 7 PERSPECTIVES AND PROPOSALS

The results of the research described in this thesis have implications for further study. In particular a number of problems, associated with the investigation of mutations of the mitochondrial genome, have been identified. These will be described and solutions discussed.

A large-scale sequencing project such as this is greatly impeded by the lack of a database of known mitochondrial mutations. As described in Chapter 1 all human mitochondrial sequence is traditionally compared to the so-called "Cambridge" sequence published by Anderson et al (1981). However, a number of differences have been reported in normal control individuals by numerous laboratories. This was commented upon by Howell et al (1992) and Marzuki et al (1991) and a need identified for international collaboration to establish which "mutations" are relatively common polymorphisms. Rare polymorphisms are disease-associated if two criteria are fulfilled. Firstly, the nucleotide change (for point mutations) must never have been identified in a normal control. It is my opinion that mutations, claimed to be associated with disease but also identified in disease-free controls, e.g. some of the LHON mutations, should not be quoted as disease-associated mutations. Very often careful consideration of the data contained in such studies reveals that the statistics are extremely questionable. A case in point is the claim by Shoffner et al (1993) that a mitochondrial DNA mutation is associated with Alzheimer disease and Parkinson disease. In order to substantiate this claim and two disease groups were combined. In addition, the control group was too small to identify the nucleotide change if it was simply a rare polymorphism. The credibility of all research of mitochondrial diseases is greatly damaged by claims of association of mitochondrial mutations with specific diseases based on questionable statistics.

Secondly, a mutation has to be identified in a second, unrelated family, with similar symptoms, for it to be considered proven that it has a part to play in the aetiology of the disease. Without an international database of human mitochondrial DNA sequence these two criteria can only be fulfilled by searches of the literature and personal communication.

In this study ,the A->G transition at np 750, when found in both patient SH and patient 31 had not previously been reported. When Prezant et al (1993) published their work identifying the np 1555 mutation in association with maternally inherited AAID their table of other differences found included the A->G transition at np 750. This change in mitochondrial DNA sequence is therefore probably a relatively common polymorphism.

If a database of mitochondrial DNA sequence existed all the mutations identified in this study could have been put into it. This would make the existence of these changes known to others and may have revealed to me that they had previously been reported. The importance of the mutation at np 7445 reported by us (Reid et al, 1994) was always questionable until it had been reported in another deaf pedigree (N. Fischel-Ghodsian, personal communication). A database would facilitate all research into the association of mitochondrial mutations with specific diseases.

One of the greatest puzzles of mitochondrial diseases is the tissue specificity of phenotypic expression observed in many of the mitochondrial mutations already identified. The mutation at np 7445 identified in this study is a good example of this.

Where heteroplasmy is the case e.g. in MELAS, the difference in tissue expression can be related to the balance between normal and mutant mitochondrial DNA. Threshold effects have been suggested to explain tissue specificity. Letellier et al (1994) carried out experiments in which they progressively inhibited the activity of cytochrome *c* oxidase, of rat muscle mitochondria, with increasing concentrations of KCN. This effectively mimics the effects of varying the degrees of a defect on the activity of the enzyme. Their results show that respiration remains nearly maximal until a low level of cytochrome *c* oxidase activity is reached. Then there is an abrupt decrease in respiration.

Where a homoplasmic mutation is present tissue specific expression could be explained by a similar mechanism involving a nuclear encoded gene product. Much of the work on mitochondrial diseases requires the hypothetical involvement of a nuclear gene to fully explain the mechanisms by which disease arises. It is likely that much of the research carried out over the next few years in the area of mitochondrial diseases will be to determine the identity of some of these nuclear genes and their products.

There is a problem in the choice of tissue used in many studies undertaken to evaluate the role of specific mitochondrial mutations with specific diseases. Those mitochondrial diseases in which there are pathological signs of muscle disease and comparable clinical symptoms pose no problem. Muscle biopsies are relatively simple to obtain (although painful) and biochemical studies of such tissue have direct relevance to the clinical symptoms.

In the mitochondrial diseases in which the major symptoms result from defects in neural tissue e.g. LHON and sensorineural deafness, it is much more difficult to study relevant tissue. In the pedigree described in Chapter 4 biochemical studies were carried out on lymphoblastoid cells (Chapter 6), although no clinical symptoms of a defect in this tissue were present. Muscle biopsies of these patients could perhaps be justified but, in my opinion, given the lack of muscle symptoms this would provide little information of value in determining the role of the mutation in development of disease.

Both the mutation at np 7445 and that at np 1555 are at positions in the mitochondrial genome involved in mitochondrial protein synthesis. Both mutations seem to result in similar clinical symptoms. The defect in both seems to be within the ear, either in the nerve (8th cranial) or in the hair cells within the cochlea. The obvious tissue of choice for thorough biochemical investigation would come from the inner ear. Cochlear implants are now undertaken and cochlear tissue is available, but these manipulations are not done on patients such as those described here. The deafness has to be much more profound and of specific aetiology before cochlear surgery would be considered. It would be unethical to embark upon such studies in patients with mild to moderate hearing loss. The problem remains: how can meaningful experiments be undertaken?

The answer must initially be in *in vitro* studies of the mechanisms of $tRNA_{ser(UCN)}$ charging (for the np 7445 mutation) and the interaction of antibiotics with the mitochondrial 12S rRNA (for the np 1555 mutation).

As described in Chapter 4 the np 7445 mutation changes the nucleotide at the -4 position of the tRNAser(ucn). This position has been identified as being the "discriminator" nucleotide in amino acid charging of tRNAs. *In vitro* work in which the wild-type tRNAser(ucn) and the mutant tRNAser(ucn) are compared for charging efficiency and specificity should help to evaluate the role of the mutation in these mechanisms. Preliminary results (H.T.Jacobs, personal communication) indicate that tRNAser(ucn) levels are depleted in the patient-derived lymphoblastoid cell-line, compared with control cells.

Other mitochondrial diseases in which specific mutations have been identified are under investigation in Dr. Jacobs' laboratory. Three projects have been undertaken, in recent years, to code correct mitochondrial genes by site-directed mutagenesis. The genes in question were ATPase 6 and 8 to study NARP, ND4 to study LHON and COI to study Kearns-Sayre Syndrome. This was done with a long-term aim of studying each disease in a transgenic-mouse model. Each of the mitochondrial genes studied in this way encode polypeptides which are part of the

respiratory chain. A similar strategy would not be possible with the two mutations now identified in association with sensorineural hearing loss. Both are in genes encoding RNAs, 12S rRNA in the case of np 1555 and tRNAser(ucn) in the case of np 7445. Mouse models would only be possible if maternally inherited deafness could be identified in mouse lineages. Identification of mitochondrial mutations at positions of significance in mitochondrial translation would allow relevant studies to be undretaken. Unlike the situation in humans , ther e would be no objections to studying a wide range of tissues from mice.

Perhaps one of the most informative studies would be a thorough neurological investigation of members of the pedigree described in Chapter 4. So far only thier audiological and visual status has been established. A more detailed investigation may reveal other neurological symptoms which have so far not been identified. Our understanding of the mitochondrial disease in this family could only be enhanced by such a study.

This is obviously work for the future. As with all scientific research interchange of ideas at local, national and international level increases the pace of work in specific areas of study. It is to be hoped that elucidation of the mechanisms by which the mitochondrial mutations identified in association with sensorineural hearing loss will progress quickly and bring benefit to the patients.

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