Errata

(Pages 4, 93, 98, 103, 108). The point mutation described in patient 21 with Leber's Hereditary Optic Neuropathy has not been confirmed. Subsequent work demonstrated this was sequencing artifact.

Page 66. This legend should read: Restriction enzyme analysis with Bam HI probe with total mitochondrial DNA.

Page 67. Restriction enzyme analysis with Aba II probe with total mitochondrial DNA.

An investigation of the molecular genetics of some disorders of the mitochondrion.

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An investigation of the molecular genetics of some disorders of the mitochondrion.

Contents

GLOSSARY OF TERMS AND ABBREVIATIONS

(NB For internationally agreed nomenclature for mitochondrial genes see appendix ll, for stock reagents see also constituents in section 5.10)

BSA: bovine serum albumen

Chl/IAA 24:1 mixture of chloroform and isoamyl alcohol

cDNAs or complementary DNAs are DNA copies of messenger RNAs (mRNAs), and hence represent the amino acid sequence of the resulting protein

CPEO: Chronic progressive external ophthalmoplegia (see section 3.3.1)

Deletion: A mutation characterised by the absence of a region of DNA present in normal individuals

DH5αF': strain of E. coli (see Raleigh et al. 1988)

dH_2O: De-ionised water

Direct duplication: duplication where the two identical DNA segments have the same orientation.

D-loop: The control region of mtDNA, containing promoters and origins of replication

dsDNA: double stranded DNA

DTT: dithiothreitol

Duplication: A mutation characterised by two identical copies of a region of DNA normally present as a single copy

EDTA: disodium ethylenediaminetetra-acetate

Heavy strand: mtDNA strand complementary to the light strand

HEPES: N-2-hydroxyethylpiperazine-N-1-ethanesulphonic acid

Heteroplasmy: more than one distinct population of mtDNA within the same individual (cf homoplasmy, see Holt et al. 1988).

Homology: similar or identical sequence

Homoplasmy: a single population of mtDNA in an individual (cf heteroplasmy)

Hybridisation: the pairing of two complementary strands of nucleic acid.

JM101 strain of E. coli (see Raleigh et al. 1988)

JM109 strain of E. coli (see Raleigh et al. 1988)

KSS: Kearn's-Sayre syndrome (see section 3.3.1)

LHON: Leber's hereditary optic neuropathy.

Light strand: Conventional term for the mtDNA strand described in the reference sequence (cf heavy strand)

ß-ME: ßMercaptoethanol

Mitochondrial genome: A single copy of the entire mtDNA sequence, the mitochondrial "chromosome".

MM: mitochondrial myopathy.

MOPS: 3-[N-Morpholono]propanesulphonic acid

mRNA: messenger RNA

mtDNA: mitochondrial DNA

Northern analysis: a technique for analysing RNA. The RNA is size separated using electrophoresis, transferred to a nylon membrane and hybridised with a radiolabelled probe.

Oligonucleotide primers: short pieces of single stranded DNA used for initiating DNA synthesis in the polymerase chain reaction.

Origin of replication: location on mitochondrial genome where DNA replication starts.

PCR: polymerase chain reaction.

Point mutation: mutation characterised by the substitution, deletion or insertion of one base (used here also to include the gain or loss of a restriction site).

Polymerase chain reaction: an enzymatic method for making copies a of a DNA segment (Saiki et al. 1986)
Polymorphisms: variations in DNA sequence which do not appear to affect function
Processing RNA: modification of newly transcribed RNAs into mature mRNAs, rRNAs and tRNAs
Promotor: the location on mitochondrial genome where transcription starts
tRNA: transfer RNA
Replication: synthesis of a second copy of a genome.
rRNA: ribosomal RNA.
Restriction enzyme: an enzyme which recognises specific DNA sequences (usually 4-6 bases in length) and cuts the DNA at or near this site.
Restriction site: Location of a restriction enzyme recognition sequence on the DNA to be cut.
Ribosomes: RNA/protein complexes where mRNA sequence is translated into protein sequence
SDS: Sodium dodecyl sulphate
Southern hybridisation: is a technique for investigating the general arrangement of a DNA segment. It is based on the ability of restriction enzymes to detect specific sequences of DNA, and to cut the DNA at these sites. After carrying out a restriction enzyme digest the DNA is size-separated by electrophoresis, transferred to a nylon membrane and probed with a radiolabelled probe whose sequence corresponds (or is complementary) to the region of interest
ssDNA: single stranded DNA
Tandem duplication: duplication where the two identical DNA segemnts are contiguous.
TEMED: N,N,N',N'-tetramethylethylenediamine
Transcription: synthesis of mRNA from the coding DNA
Translation: the process whereby the mRNA sequence directs protein synthesis
tRNA: transfer RNA

ABSTRACT

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An investigation of the molecular genetics of some disorders of the mitochondrion.

Mitochondrial DNA is exclusively maternally inherited, and encodes a proportion of the subunits of the electron transport chain. As some of the mitochondrial myopathies show a maternal pattern of inheritance and/or a biochemical defect of respiratory chain components, they could be caused by mutations in the mitochondrial genome. Leber's hereditary optic neuropathy could arise in the same way, as it is maternally inherited.

The main aim of this project was to determine whether mutations of mitochondrial DNA are responsible for these diseases. During the period of the study, rearrangements of mitochondrial DNA were described in mitochondrial myopathy (Holt et al. 1988b), Leber's hereditary optic neuropathy (Wallace et al. 1988a) and Pearson's syndrome.

Restriction enzyme analysis of mitochondrial DNA from blood using five enzymes in 10 patients with mitochondrial myopathy did not reveal any major rearrangements in 83% of the protein coding regions. Similarly, the only point mutations in the 5% of this region which comprised the restriction sites were probably population polymorphisms. Ultimately 3/10 patients, all of whom had external ophthalmoplegia, were shown to have mitochondrial DNA deletions in muscle which were not readily detectable in blood. The polymerase chain reaction was then applied in order to detect abnormal mtDNAs which might be present in the blood of 24 patients, with a view to developing this method for noninvasive diagnosis. A product of appropriate size was obtained in 8. These observations confirmed that low levels of deleted mtDNAs are present in blood of a substantial proportion of patients, and in two asymptomatic relatives. Sequence analysis suggested that mitochondrial DNA deletions were present in the germline of this family.

Two patients with direct tandem duplications of mitochondrial DNA and mitochondrial myopathy are described. The breakpoint regions between duplicated segments were amplified using the polymerase chain reaction, cloned and sequenced. The distribution of normal and abnormal genomes in different tissues was investigated using Southern hybridisation, and in different cells within the same tissue using PCR. In each case the gene for cytochrome

oxidase subunit I (MTCOX1) was interrupted, creating reading frames which if transcribed and translated would result in truncated versions of this peptide. Heteroplasmy and mosaicism for the abnormal mtDNA population was apparent.

Nineteen patients with Leber's Hereditary Optic Neuropathy were investigated for the presence or absence of the point mutation described by Wallace et al. (1988a), using Southern hybridisation, and sequencing, oligonucleotide probing and competitive oligonucleotide priming of PCR products. In 14 cases the point mutation was confirmed, and 5 had the wild-type sequence in this region. Of the 5 patients with the normal sequence, only one had a clear family history of LHON. Sequence analysis in this patient revealed a single base insertion between bp 11,718 and 11,719 producing a frame shift. If transcribed and translated this would result in a truncated peptide. This provides evidence that Wallace's mutation is present in the majority of patients with LHON, and support for the view that both mutations are causative.

These observation support the contention that mutations of mitochondrial DNA may cause disease. Further work may provide insights into the mechanism by which they cause the phenotype, and suggest therapeutic possibilities. The use of the polymerase chain reaction to detect deletions in blood may enable non-invasive diagnosis in up to 40% of patients with mitochondrial myopathy and identification of relatives at risk of transmitting the disease.

Chapter 3: Introduction

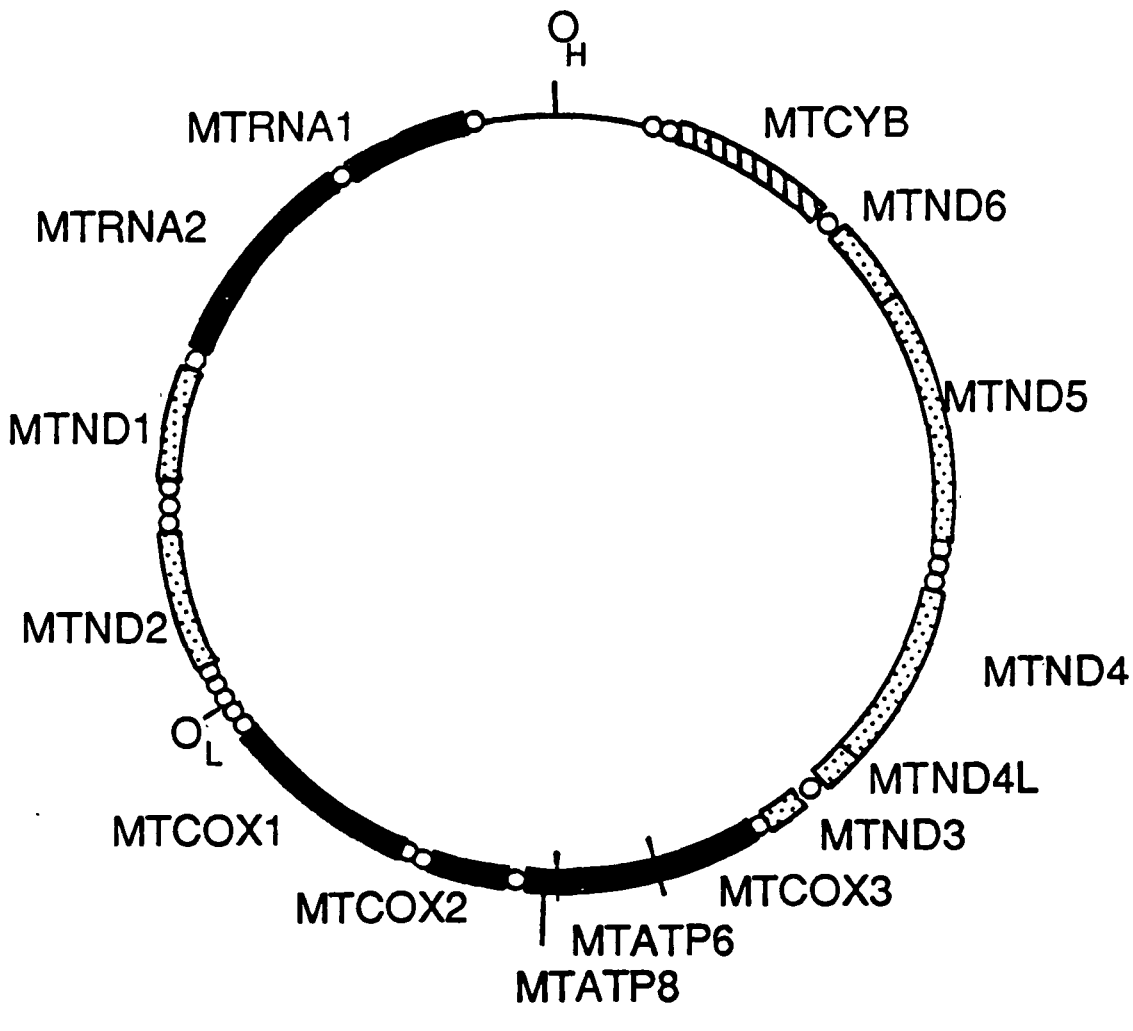
NB: Please see glossary for abbreviations and appendix 11 for mitochondrial gene nomenclature

In the past decade molecular techniques have transformed clinical genetics (Weatherall 1985). Many mutations causing metabolic diseases have now been characterised, enabling a better understanding of disease pathogenesis. The mitochondrial myopathies have previously been diagnosed on the basis of abnormal skeletal muscle biopsy (Petty et al. 1986): light microscopy shows ragged red fibres on Gomori Trichrome staining (clumps of mitochondria around the periphery of the muscle cell), and abnormal mitochondrial morphology on electron microscopy (often large mitochondria with abnormal cristae and paracrystalline inclusions). Suggestions that they might be caused by mutations of mitochondrial DNA (mtDNA) were based on their predominantly maternal pattern of inheritance, and their association with defects of the respiratory chain. This was the major hypothesis to be tested when this work was initiated. Since that time, both major re-arrangements and point mutations of mitochondrial DNA (mtDNA) have been associated with human disease (Wallace 1989, Holt et al 1988b, Poulton et al 1989a, Wallace et al. 1988a, Rotig et al. 1988). The mechanisms whereby these mutations arise and impair mitochondrial function remain unclear. Nevertheless it is likely that in future the clinical classification will become simpler and revolve around the DNA analysis, and that it will become important in diagnosis as well as genetic counselling. In this introduction, I will first describe the organisation of mtDNA, its pattern of inheritance and mutations in other animals, and then outline the clinical syndromes and the mutations associated with human disease.

3.1 Mitochondrial DNA

3.1.1 The organisation of mitochondrial DNA

Mitochondria are intracellular organelles which are key elements in oxidative phosphorylation. Their inner membrane binds the four complexes of the electron transport chain (complexes I to IV respectively, Attardi and Schatz 1988), and ATP synthase. Mitochondria contain their own DNA, which is the only source of extranuclear DNA in man (reviewed in full, Attardi 1984). Unlike es.



NORMAL

Legend to Fig 3.1

The normal mitochondrial genome.

KEY Circles represent genes for transfer RNAs, O_H: Origin of replication of the heavy strand. O_L: Origin of replication of the light strand. MTATP6 and 8: subunits 6 and 8 of ATP synthase, MTCOXI to III: subunits I to III of cytochrome oxidase, MTCYB: cytochrome b, MTND1 to 6 and 4L: subunits 1 to 6 and 4L of NADH dehydrogenase.

nuclear DNA, there are thousands of copies of this 16 kilobase circular molecule in every nucleate cell, each mitochondrion containing between two and ten copiThe human mitochondrial genome was sequenced in 1981 by Anderson et al., and each copy encodes thirteen proteins, 22 transfer RNAs (tRNAs) and two ribosomal RNAs (Fig 3.1). The proteins are all subunits of the electron transport chain (ie subunits 1 to 6 and 4L of complex I (Chomyn et al. 1985), apocytochrome b from complex III, subunits I to III of complex IV and subunits 6 and 8 of ATP synthase as shown in table 3.1). Mitochondria require their own ribosomes and transfer RNAs (tRNAs) because they have their own version of the genetic code. The mitochondrial genome is extremely compact with most of the genes abutting, and only just over 1kb of non-coding DNA (the so-called D-loop). This is an important control region, containing both the origins of replication of mtDNA (O_H and O_L from which synthesis of the daughter light and heavy strands start), and the promoters for RNA synthesis (LSP and HSP corresponding to light and heavy strand transcripts). The internationally acepted abbreviations for all mitchondrial genes are listed in appendix 11.

3.1.2 Transcription of mtDNA

Unlike nuclear DNA, the genes are transcribed in two long polycistronic (continuous) RNAs, one in each direction (the so-called heavy and light strand transcripts being the major and minor coding strands respectively, see Attardi 1984). The RNA polymerase requires a specificity factor, mtTF1, which has been purified and characterised by Clayton et al. (1988). Two distinct transcripts are synthesized on the heavy strand, the majority terminating at the boundary of MTRNR2 and MTTL1 (see appendix 11 and fig 3.1), and a termination factor which may cause transcription attenuation has been identified (Kruse et al. 1989). The longer H strand transcript and the L strand transcript are then processed (that is cleaved into pieces corresponding to the genes they encode and modified), resulting in mature mRNAs, tRNAs and ribosomal RNAs (rRNAs). The molecules required for this processing have not yet been identified.

3.1.3.Replication of mtDNA

This has been reviewed by Clayton 1987 and Litvak et al. 1988. There are two origins of replication: the origin for the heavy strand (O_H) in the D-loop,

and the origin for the light strand (O_L) in a small non-coding region in a cluster of tRNAs approximately 8kb away. Replication of mtDNA is initiated at O_H, by a gamma DNA polymerase, primed by an RNA transcript initiated at the LSP. Those transcripts which are not destined to become mRNA are cleaved by the mitochondrial RNA processing activity (RNase MRP) exactly at the position corresponding to the 5' end of the nascent DNA strand. Interestingly, this endoribonuclease contains an RNA component with probable homology to the nuclear RNAse P (Gold et al. 1989). Replication of the light strand is not initiated until the replication fork has gone round 2/3 of the genome and reaches O_L, where the origin is probably made available to the DNA polymerase by the formation of a hairpin-like structure. DNA synthesis is primed by a short stretch of riboadenosines synthesized by an RNA primase. Thus for much of the estimated one hour required to synthesize the daughter strands, there is a variable length of heavy strand left unpaired and potentially vulnerable to recombination (see chapter 10). Gapped daughter molecules are then converted to closed circles, and negative superhelical turns introduced by a DNA gyrase.

Thus, there are a large number of subunits of the electron transport chain, enzymes required for mitochondrial replication, transcription, translation and assembly of the mature electron transport chain which are NOT encoded by mtDNA. As all of these nuclear encoded products must be transported into the mitochondrion, respiratory function could be impaired by nuclear mutations involving their coding regions, synthesis, transport or assembly. There are examples of many if not all of these types of mutants in yeast (Grivell 1989), and it is likely homologues for some of them exist in mammals.

3.1.4.Homoplasmy and inheritance of mtDNA

The majority of the thousands of millions of copies of mtDNA in each individual are identical (so-called homoplasmy). This was laboriously demonstrated by Monnat and Loeb (1985) who sequenced 80 kilobases of mtDNA from 387 clones of the same region from 4 patients (80kb). For each individual all the clones except three were identical, and these differed at a single base position each, by one or two base pairs. Thus although mtDNA mutations probably accumulate at low levels in somatic cells with senescence (Piko et al. 1988), the majority of mtDNAs in most humans are identical to their mother's. Clearly,

in order for new mtDNA polymorphisms (variations in DNA sequence which do not appear to affect function) to arise and be transmitted, the mtDNA mutation must occur in the germline. Although each ovum contains many thousands of mtDNAs, the genomes which ultimately populate the organism appear to arise from only a few or a single mitochondrion (the so-called "bottle-neck" hypothesis, Hauswirth and Laipis 1985,). Thus all of the millions of mtDNAs in a mother may differ by a single base pair from that of their offspring. Because recombination of mitochondrial DNA is a rarity (and indeed all the examples so far described in man have been associated with disease), the accumulation of mtDNA mutations has been used by population geneticists as a "genetic clock" (Cann et al 1987). All mtDNA types so far described probably arise from a single African type around 200,000 years ago (the "mother Eve hypothesis", reviewed Poulton 1987). This type of study shows that the majority of point mutations are "silent" because they occur in the third base position, that transitions occur more frequently than transversions, and that the mutation rate of coding regions of mtDNA is about ten times that of nuclear genes (Wallace et al. 1987). Furthermore, length polymorphisms differ by only short sequences, for instance the 9 bp sequence in the non-coding region between COI1 and $tRNA^{lys}$ may be duplicated (Wrisnick et al. 1987) or deleted (Hertzberg et al. 1989).

3.2 Mutations of mitochondrial DNA in other organisms

Mitochondrial genomes vary widely across the animal and plant kingdoms, and a large number of fascinating processes occur which are of doubtful relevance to the problem of mtDNA in human disease.

3.2.1 Size and general arrangement of mtDNA in other organisms.

The mammalian mitochondrial genome is at the lower end of the size range for mitochondrial genomes. At 16.5 kb it is extremely compact with the minimum of non-coding DNA. In contrast, mtDNA averages 70 kb in yeast (Bockelmann et al. 1986) and may reach 2,400 kb in higher plants (Butow 1986). The "non-coding" DNA may contain pseudogenes, duplications and even fragments homologous to chloroplast DNA in plants, while introns may be self-splicing in yeast or encode endonucleases. While all mt genomes appear to contain tRNA genes, in various protozoa some or all of the functioning mitochondrial tRNAs are nuclear encoded

and transported into the mitochondrion (Attardi and Schatz 1988). The numbers of mitochondria per cell may vary from unity in some unicellular organisms to hundreds of thousands in a mammalian oocyte (Hauswirth and Laipis 1985), and mtDNAs per mitochondrion may change according to the stage of development. Although re-arranged mitochondrial molecules have only been found in association with disease in humans, a range of sub-genomic circles are the norm in some plants ("sublimons", Butow 1986), presumably derived from the master mitochondrial genome, which is itself present at very low levels (Bendich, 1990). Small et al. (1989) have recently provided evidence that evolution of the 570kb master mitochondrial genome in maize may occur via recombination of these sub-genomic circles. Furthermore, mitochondrial plasmids are closely associated with senescence in fungi (Dujon and Belcour 1989).

3.2.2 Heteroplasmy and length polymorphisms

Of particular relevance to the finding of rearrangements of mtDNA in human disease is the question of whether heteroplasmy with deletions/duplications are necessarily pathological. Do they occur as population polymorphisms in other species? This section will first attempt to answer this, and then mention three groups of mutations with well established phenotypic effects: maternally transmitted antigen (MTA) in mouse, petit mtDNAs in yeast and cytoplasmic male sterility in plants.

Although heteroplasmy is not a normal finding in man or cows (section 3.1.4) it has occasionally been found in many species, and it is not uncommon in others including Drosophila (Solignac et al. 1987) fish (Bentzen et al. 1988) and fungi (Dujon and Belcour 1989). Neither is it confined to organisms only distantly related to man: it occurs as a population polymorphism in the domestic rabbit. Mignotte et al. (1990) have recently described tandem repeats 20bp in length in the D-loop of the domestic rabbit. The number of repeats varies from one to ten both among members of a sibship and within an individual. It is possible that these are generated by polymerase slippage during replication, although more than one type may be transmitted to each individual in the germline. Similarly, heteroplasmy for a shorter length polymorphism has been described in cows (Hauswirth et al. 1984). This comprises variation of a few bases in the homopolymeric deoxycytidylic acid tract just downstream of the

origin of replication of the heavy strand, and close to the conserved sequence blocks which are probably involved in processing the RNA transcript which primes replication. This variation has been described in human cell lines (Hauswirth and Clayton 1985), but it is not known whether this is a normal finding in humans. Boursot et al. (1987) described two mice who were heteroplasmic for large deletions of mtDNA, but this is a rare occurrence, and it is not certain whether the mice were symptomatic. There are no other known instances of heteroplasmy for mtDNA species differing in length by 5-8kb as found in human disease, as a normal finding in a closely related species.

The homoplasmic length polymorphisms which have been described in man (section 3.1.4) are confined to non-coding DNA and are only a few base pairs in length. The nearest example of a length polymorphisms of comparable size to those described in human disease is in homoplasmic parthenogenetic lizards. Moritz and Brown (1987) have described tandem duplications up to 8kb in length and occurring mainly in the D-loop region. As they occur significantly more commonly in triploids than diploids, their presence may be related to the nuclear background.

3.2.3 Some mitochondrial mutations with phenotypic effects.

Not only can nuclear genes affect expression of mitochondrial genes (section 3.1), but mitochondrial gene products can affect the expression of nuclear genes in both yeast (Parikh et al. 1986) and mammals. The phenomenum of maternally transmitted antigen (MTA) in mice is an example of this. The expression of the MTA cell surface antigen encoded in the nucleus requires both $ß_2$macroglobulin and one of a group of mtDNA types (Rodgers et al. 1986). Although a number of point mutations appear to be associated with these various mtDNAs, so far there is no single characteristic change or mechanism of action. It may be that mitochondrial translation products are transported out of the mitochondrion and interact with these nuclear gene products at the cell surface.

Large deletions of mtDNA in yeast are associated with the so-called petit phenotype (recently reviewed by Dujon and Belcour 1989). Not only do the colonies have an abnormal appearance, but aerobic respiration is impaired. In some of these the remaining mtDNA includes promotors and protein reading frames, and transcription can be demonstrated, although translation of mitochondrial RNAs

does not occur. There is some evidence that the phenomenum of suppression, the ability of a rho^- genomes to replace the wild type in strains where both are present, depends on the number of origins of replication of per mutant genome (De Zamaroczy et al. 1981). Thus it would appear that increasing the number of origins of replication per unit length of mtDNA might result in some kind of "replicative advantage". However, this must be an oversimplification, as in some suppressive strains the mtDNA comprises repeats of short simple sequences with no recognisable origins of replication, but replication still occurs. Very recently, Fangman et al. (1990) have described a mutation, hsr 1 in the nuclear gene, HSR 1 (for hypersuppressive response) which is a nuclear recessive. This appears to influence the expression of hypersuppression: it reduces the rate at which pure rho^+ and pure rho^- mtDNA types segregate. It may therefore control some essential step in mitochondrial or cell replication which acts differentially on rho^+ and rho^- mtDNAs.

Cytoplasmic male sterility (CMS) in plants is a maternally inherited trait which results in the lack of pollen production. There are three distinct types of CMS in maize depending on their nuclear restorer gene. The CMS-T cytoplasm is associated with a novel mitochondrially encoded 13kd polypeptide, arising from an open reading frame (Dewey et al. 1988) which inserts in the mitochondrial membrane. This determines susceptibility to the fungal pathogen Bipoaris maydis, which secretes toxin BmT, which is able to bind to the 13kd protein and induce respiratory depression and massive ion leakage out of the mitochondrion (Braun et al. 1990). There is an example of a mitochondrial gene associated with CMS in Petunia which comprises duplications of parts of MTCOX11 and the gene for ATP subunit 9 fused to an unidentified reading frame (Young and Hanson 1987). Both of the former contain a number of other re-arrangements. This gene is transcribed, and if translated would result in an abnormal truncated cytochrome oxidase subunit 11.

3.3 Mitochondrial myopathy

There are many conditions in which mitochondria may occasionally have abnormal morphology, such as ischaemia, chronic alcohol ingestion, Retts syndrome etc. Probably only a small proportion are caused by mitochondrial mutations. Because mitochondrial DNA (mtDNA) is exclusively maternally inherited (Giles et

al. 1980) and encodes subunits of the electron transport chain, diseases caused by mutations in mtDNA would be expected to have a maternal inheritance pattern and be associated with defects in mitochondrial metabolism.

The mitochondrial myopathies are a heterogenous group of disorders, some of which fulfil these criteria (Egger and Wilson 1983, Petty et al. 1986). Furthermore, major rearrangements of mtDNA have recently been described in up to 40% of patients (Moraes et al. 1989b), and more recently a point mutation, which may be causative in one family with a mitochondrial myopathy-like illness (Harding 1989).

This subsection will summarise the main clinical presentations and biochemical defects in this group of disorders, and then describe the patterns of inheritance and the rearrangements of mtDNA associated with certain clinical phenotypes.

3.3.1 Clinical syndromes of mitochondrial myopathy

Much has been written about the clinical and biochemical classification of this group of disorders, but in practice many patients do not exactly fit into any one syndrome. It is to be hoped that DNA analysis will rationalise the approach to classification.

The major clinical syndromes described can be divided according to age of onset into those presenting either from middle childhood onward, and those presenting in infancy. The first of these two groups primarily affect muscle and brain, and have been divided into: Kearn's-Sayre syndrome (and associated variant, chronic external ophthalmoplegia), MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis and strokes, Pavlakis et al. 1984) and MERRF (myoclonic epilepsy and ragged red fibres, Fukuhara et al. 1980), in which characteristic muscle biopsy is a prerequisite. However, tissues other than muscle, brain and nerve can also be involved, resulting in multisystems disease: cardiomyopathy, renal tubular defects, growth retardation with or without growth hormone deficiency, diabetes and bone marrow suppression have all been described.

The clinical presentations have been reviewed by DiMauro et al. 1985, who argue that their features are sufficiently different to be distinctive syndromes. Kearns-Sayre syndrome is defined as the triad of onset below age 15

years, progressive external ophthalmoplegia, pigmentary degeneration of the retina, plus one of the following: heart block, cerebellar ataxia or high cerebrospinal fluid protein. Chronic progressive external ophthalmoplegia (CPEO) is the term usually applied to patients who do not quite fulfil all these criteria. MERRF usually presents as myoclonus, and ataxia, weakness and generalised seizures. MELAS may have growth retardation, episodic vomiting, seizures and recurrent stroke-like episodes causing hemiparesis, hemianopia or cortical blindness. Petty et al. (1986) found no correlation between biochemical defect and clinical syndrome in their series of adults. However, in a more recent review, DiMauro et al. (1989) reclassify the clinical syndromes by biochemical defect. While there may certainly be a wide range of symptoms even within a pedigree where the mutation is presumably identical (Ishitsu et al. 1987), it is likely that some of this difference in emphasis relates to the differences in diagnostic methods used in various centres.

While any of the above syndromes may present in childhood, there are a number of clinical presentations which are primarily found in children rather than adults (DiMauro et al. 1989). Many of these are associated with cytochrome c oxidase deficiency, and once again DiMauro divides them into groups: those dominated by muscle disease, and those dominated by encephalomyopathy (the latter including a proportion of the patients with Leigh's encephalopathy, see below). The patients with cytochrome oxidase deficient myopathy present in the first few months with hypotonia, weakness and lactic acidosis, followed by respiratory insufficiency. Death by the age of six months is the usual pattern in the so-called "fatal" group, whereas those in the "benign" group improve and are normal by 3 years of age. There is also a group of patients with cardiomyopathy often associated with complex 111 deficiency.

3.3.2 Deficiencies of specific subunits in mitochondrial myopathy

Much of the work on the mitochondrial myopathies has been hampered by initial difficulties in obtaining cell lines which accurately reflected the biochemical defects found in vivo. Recently, both fibroblast (Robinson et al. 1987) and myoblast lines (Shimoizumi et al. 1989) have been selected, and this has enabled pulse labelling of mitochondrial translation products with ^{35}S-methionine (Bolhuis et al. 1988). So far, however, very few abnormalities in mitochondrially encoded subunits have been identified. Bolhuis et al. 1988

Table 3.1: Respiratory chain components

Complex	Subunits encoded by mtDNA		No. of nuclear-encoded subunits	Total no subunits	Active components: cytochromes/co-enzymes/prosthetic groups
	Proteins	Genes			
I	ND1 ND2 ND3 ND4 ND4L ND5 ND6	MTND1 MTND2 MTND3 MTND4 MTND4L MTND5 MTND6	19	25	Flavin mononucleotide 6 or 7 non-haem iron-sulphur centres Coenzyme Q
11	none	none	4 or 5	4 or 5	Flavin adenine dinucleotide Nonhaem iron-sulphur centres Coenzyme Q
111	apoCytb	MTCYB	8 or 9	9 or10 10	Cytochromes b,c c_1 Protoporphyrin 9
lV	COl COll COlll	MTCOXl MTCOXll MTCOXlll	10	13	Cytochromes a and a_3 Haeme A 2 copper atoms
V	ATPase6 ATPase8	MTATP6 MTATP8	10 to 12	12 to 14	

identified an abnormal ND3 subunit in a patient with complex 1 deficiency (apparent molecular mass 13,100 versus 13,700 in controls). However, when the gene was sequenced, no abnormality was found (Attardi et al. 1990), and it was concluded that there must be another defect, for instance in RNA processing. Furthermore, immunoblotting of Western blots of purified mitochondrial protein have been generally unable to identify abnormal mitochondrially encoded subunits appropriate to the specific biochemical defect. Using antibodies to complex 1, Schapira et al. (1988) found either a normal pattern, or a global reduction inall subunits, or a specific reduction in nuclear encoded subunits containing iron-sulphur prosthetic groups. More recently, Holt et al. (1989c) found no abnormality in most patients known to have mtDNA deletions, and deficiencies of both mitochondrial and nuclear encoded subunits in some patients in whom mutations had not been identified.

There are several reasons why this type of study have been relatively unhelpful in elucidating mtDNA mutations. Firstly, there may be local specific deficiencies which cannot be detected when the sample contains both normal and abnormal mitochondria, and require either clonal cell lines with very high proportions of abnormal mitochondria or immuno-histochemistry for detection. Secondly, some point mutations may not affect the mobility of the peptide or alter the epitope to which the antibody is directed, and may therefore appear normal. However, as more mutations are characterised and specific antibodies raised, it seems likely that this type of study will eventually help clarify the pathophysiology of diseases causes by mutations in mtDNA.

3.3.3.Patterns of inheritance

The majority of the cases of mitochondrial myopathy (MM) are sporadic. Around 20% have a positive family history, and where this is present maternal transmission exceeds paternal transmission, with no excess of affected males as would be predicted of inheritance were X linked. Thus in the series by Petty et al. (1986), 3/10 familial cases were mother-child pairs, in 3 further families affected individuals were maternally related, 3 were sib pairs and in only one was there paternal transmission. In a more recent review the ratio of maternal to paternal transmission was 58:8 (Harding AE, personal communication). Although occasional large pedigrees exist where maternal transmission is clear

(Rosing et al. 1985), it is unusual for all offspring of an affected mother to be clinically involved, and the severity and age of onset may be variable within a pedigree. The risk to offspring of an affected mother has been estimated as 5.5% and the risk to sibs 3% (Harding et al. 1988).

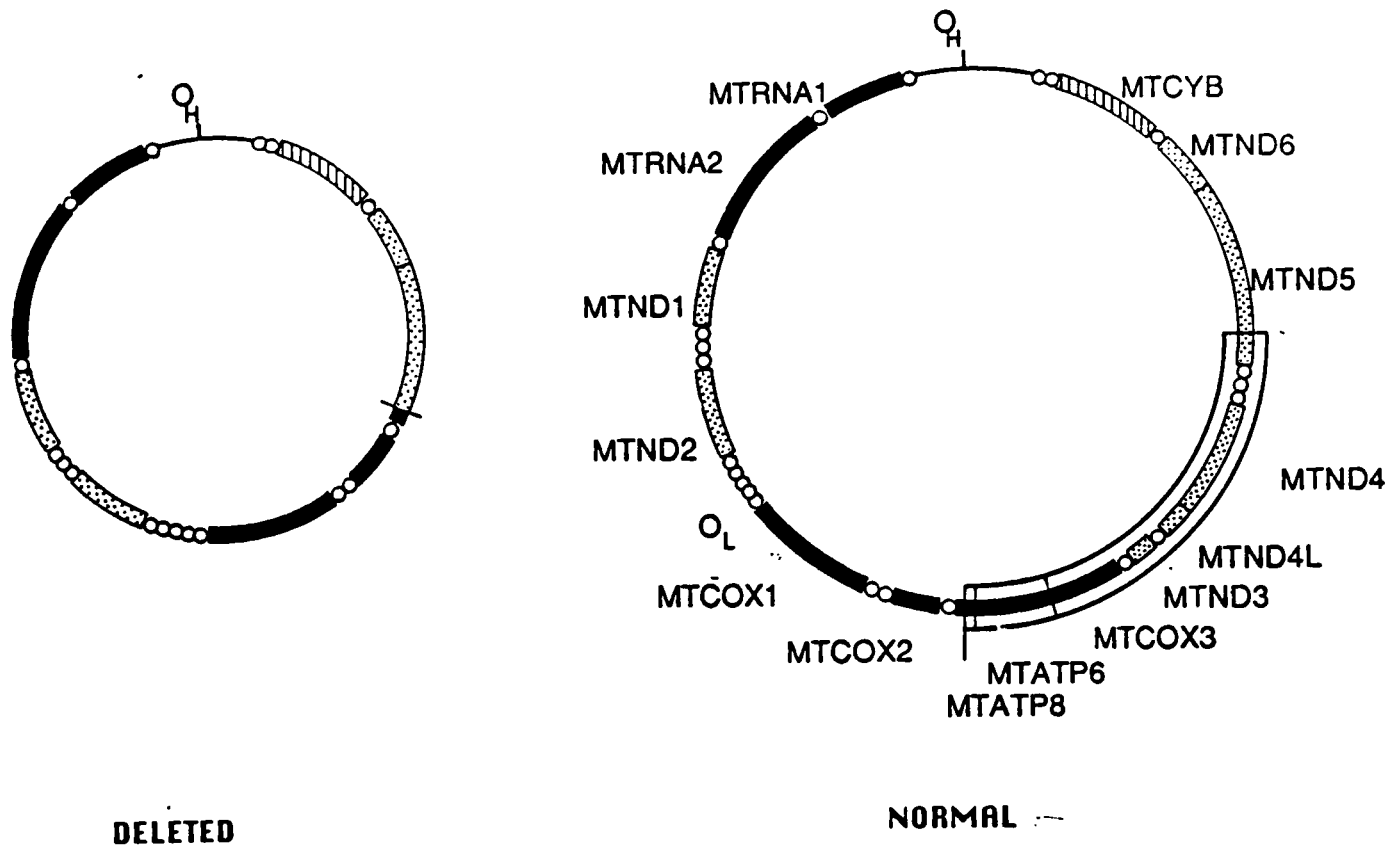
The finding of a predominantly maternal inheritance pattern in familial cases is perhaps unexpected for two reasons. Firstly, the majority of the subunits of the electron transport chain are actually encoded in the cell nucleus, including the iron sulphur binding sites shown to be selectively reduced in some patients (Moreadith et al. 1987, Schapira et al. 1986). Causative nuclear mutations should result in classical autosomal recessive, autosomal dominant or X linked patterns of inheritance. This suggests that nuclear mutations are probably in a minority. Secondly, as there are age and tissue specific isoforms of these nuclear encoded mitochondrial enzymes (eg both cytochrome oxidase (Kadenbach et al. 1986) and ATP synthase (Gay and Walker 1985)), it is easy to see that mutations of nuclear genes could cause the tissue specific and developmentally regulated biochemical defects which have been described. On the other hand, it is not immediately obvious how mitochondrial mutations could give rise to the complexities observed. Although this has not yet been clarified, heteroplasmy (more than one distinct population of mtDNA within the same individual, see Holt et al. 1988) and mosaicism (different ratios of normal and abnormal genomes in different tissues) can be used to explain several features (see below and Wallace et al. 1988b).

3.4 MtDNA mutations in human disease.

No nuclear mutations have yet been identified in association with MM, although they have been inferred (see subsection 3.4.1 below and Zeviani et al. 1989a). Mitochondrial mutations can be divided into deletions, duplications, complex and point mutations. Each of these will be considered in turn.

3.4.1 Deletions of mitochondrial DNA

The first evidence that rearrangements of mtDNA might cause human disease was obtained by Holt et al. (1988b) who used Southern hybridisation (Southern 1975), to demonstrate large deletions in mtDNA from muscle, which were not detectable in blood (this is heteroplasmy, or the existence of more than one



Legend to Fig 3.2

The common deletion alongside the normal mitochondrial genome drawn to scale. Boxed region represents the deleted region.

KEY Circles represent genes for transfer RNAs, O_H: Origin of replication of the heavy strand. O_L: Origin of replication of the light strand. MTATP6 and 8: subunits 6 and 8 of ATP synthase, MTCOXI to III: subunits I to III of cytochrome oxidase, MTCYB: cytochrome b, MTND1 to 6 and 4L: subunits 1 to 6 and 4L of NADH dehydrogenase.

mitochondrial type in the same individual). Although circumstantial evidence is accumulating that these are causative, the pathophysiological mechanism still obscure. These findings have now been confirmed by a number of groups (Lestienne et al. 1988, Zeviani et al. 1988, Nelson et al. 1989, Larsson et al. 1990, Johns and Hurko 1989), and it is now established that deletions may be demonstrated by Southern hybridisation of muscle mtDNA in virtually all patients with KSS (Holt et al. 1989c, Moraes et al. 1989b), and in a proportion of patients with Chronic Progressive External Ophthalmoplegia (CPEO). Unexpectedly, these cases are generally sporadic, and in many there is no associated specific biochemical defect other than a high proportion of cytochrome oxidase (COX) negative fibres. In the next section, I will firstdescribe the deletions which have been observed in sporadic cases, then explore the relationship of the deletions to the phenotype.

The "common deletion"

All the deletions described so far include one or more tRNA genes and part or all of one or more protein reading frames (except for one as yet unconfirmed report by Saifuddin Noer et al. 1988). All except one (Holt et al. 1988b) lie within the approximately 10kb of DNA separating but excluding the two origins of replication. The deletion found most frequently, the so-called "common deletion" (fig 3.2) comprises approximately 50% of most series of deletions (Holt et al. 1989). The following genes are deleted: MTATP 6, MTCOXIII, MTND3, MTND4L, MTND4, parts of MTATP 8 and MTND5 and 5 tRNA genes (see fig 3.2). Sequence analysis (Schon et al. 1989a, Holt et al. 1989a) reveals that there are often exact homologies at either end of the deleted segment of up to 13bp as occurs in the common deletion (fig 10.1). Furthermore, it appears that this particular stretch may be a recombination "hot spot" as a number of deletions share one end or the other.

How do these rearrangements match up to the clinical features? Surprisingly, Moraes et al. (1989b) found no clear relationship of the phenotype either to the region of the mitochondrial genome deleted, the length of mtDNA deleted (1 to 7kb) or the percentage of mitochondrial DNAs which are deleted (which may range from 20 to 80% in muscle). The identical deletion may give rise to varied phenotypes, mild or severe. Where a number of biopsies have been

taken from the same individual the proportion of deleted genomes increases with time, consistent with clinical deterioration (Larsson et al. 1989). In all cases of MM with mtDNA deletions described so far, the tissue distribution is similar in outline: the highest proportion is found in muscle (and where multiple biopsies have been taken from different sites in the same individual they are not significantly different), but deleted DNAs may be found in other tissues such as brain, liver, kidney and blood. Some investigators have reported that deleted genomes are confined to muscle in patients with CPEO (Zeviani et al. 1989b), but may be distributed more widely in KSS, consistent with their clinical involvement. Thus, there may be a dosage effect: clinical severity increases with increasing proportion of deleted genomes within an individual or tissue.

Autosomal dominant deletions

The first familial cases of mtDNA deletions associated with mitochondrial myopathy were not maternally inherited as had been predicted, but autosomal dominant. The first published case was a mother and daughter with deletions whose ends mapped to different regions of mtDNA (Ozawa et al., 1988). Zeviani et al. (1989a) described a family with autosomal dominantly inherited tendency towards deletion: all individuals carried a number of distinct deletions, and no family member shared any deletion with any other. They suggested that the deletions occurred de novo in each individual. As one end of all the deletions mapped to the same location within the D-loop or control region (the other end accounting for almost all the variation), it is possible that these are caused by a failure of mechanisms involved in normal replication.

It has been suggested that variable deletions of mtDNA also occur in conditions as diverse as hypertrophic cardiomyopathy and recurrent myoglobinuria on the basis of PCR products (Ozawa et al. 1989). This has now been supported with sequence data (Ozawa, personal communication). No abnormality was apparent on Southern hybridisation, suggesting that any deletions must be present at very low levels.

3.4.2 Rearrangements in haematological disorders

Pearsons syndrome (Pearson et al. 1979) is an unusual haematological disorder presenting in infancy with sideroblastic anaemia, exocrine pancreatic dysfunction and metabolic acidosis. More recently, hepatic dysfunction has also been noted as a prominent feature (Rotig et al. personal communication). Although the major symptoms of this disorder do not arise from muscle involvement, there is good reason to suppose that it is closely related to the mitochondrial myopathies. Firstly, the rearrangements of mtDNA which have been reported are similar, for example, the "common deletion" has been found in both disorders (Rotig et al. 1988 and 1989a). However, the proportion of deleted genomes was generally higher and more uniform (ie both populations easily detectable by Southern hybridisation) in all tissues examined than has been found in the mitochondrial myopathies (Munnich 1989). Typical values would be 80% and 50% deleted genomes in blood and muscle respectively. In contrast, patients with mitochodrial myopathy rarely have as much as 10% deletions in blood. Secondly, one case of mitochondrial myopathy (KSS) has been reported who had suffered a Pearson's-like illness in early childhood (Larsson et al 1990).

3.4.3 Point mutations

The first human disease to be associated with point mutations of mitochondrial DNA was Leber's Hereditary Optic Neuropathy (LHON) (Wallace et al. 1988a). As this form of inherited optic atrophy has no clear diagnostic clinical features it is usually a diagnosis of exclusion. Visual impairment occurs in adolescence or early adulthood, and progresses over days weeks or months to optic atrophy. There is little if any subsequent recovery and the second eye usually follows the same course within nine months, if it was not affected simultaneously. It may be accompanied by cardiac conduction defects (Nikoskelainen et al. (1985) found ECG evidence of accessory conduction pathways in 30% of patients or LHON family members), and the central nervous system is occasionally involved. LHON is maternally inherited, and is commoner in males than females. Histology reveals a vasculopathy, and both endothelial cells and occasionally muscle may contain structurally abnormal mitochondria.

In the past, both the mitochondrial enzyme, rhodanese, and cigarette smoking (causing cyanide toxicity) have been implicated in some patients (Wilson

1982). However, studies of enzyme activity in various tissues gave conflicting results (Poole and Kind 1986 found low levels of rhodanese in rectal biopsy from LHON patients, but Nikoskelainen et al. 1984 found it to be normal in skeletal muscle). Whitehouse et al. (1988) suggested that this could be attributed to the existence of tissue isoenzymes which they demonstrated using polyacrylamide gel isoelectric focusing. In a study of three patients with LHON, Whitehouse et al (1989) found no deficiency of specific isoforms in liver compared to controls. However, until each of the different isoforms has been investigated independently in more tissues in a larger series of patients, the role of rhodanese remains unclear. As all of the mtDNA reading frames has now been assigned, it is likely that these isoenzymes are encoded by a number of nuclear genes. If so, they are probably not the primary defect in LHON, although they could interact with mitochondrial gene products in causing the phenotype. So far, there is only one report of LHON accompanied by respiratory defects (Parker et al. 1989), and this was in a family with an unusual phenotype, possibly overlapping with mitochondrial myopathy (Harding 1989a). Thus, unlike the pattern of inheritance, the biochemical evidence does not support the suggestion that this might be caused by a mutation in mtDNA.

Wallace et al. (1988a) found a G to A transition at base pair 11778 in the majority of families with this disorder (9/11), and not in controls. This resulted in the substitution of a conserved arginine with a histidine in subunit four of NADH dehydrogenase. Furthermore, mitochondrial DNA typing of two pedigrees from different ethnic groups showed that despite a large number of other differences, both had this apparently LHON-specific mutation (Singh et al. 1989). Comparisons with other more closely related clans suggested that the mutation had occurred independently in each of these mitochondrial lineages since they originally diverged between approximately 40 and 80,000 years ago. Since this original finding, other workers have confirmed that the mutation is also present in a high proportion of patients (Vilkki et al. 1989 and Holt et al. 1989). However, it is still not clear how this alteration gives rise to the phenotype, or how to account for the minority of patients in whom this is not detectable nor the sex ratio of affected individuals. Holt et al (1989b) have recently shown that these patients are heteroplasmic, and that there appears to be a dosage effect: males with high levels of abnormal genomes are more likely

to have severe symptoms than those with low levels or females.

Point mutations in a mitochondrial myopathy-like disease.

One family with a point mutation of mtDNA and myopathy have recently been described by Holt et al. (1990). They did not classify these as mitochondrial myopathy as ragged red fibres were not a feature. As in the case of LHON, a single base change results in a substitution of a conserved amino acid. Although causality is less clearly established than for LHON, once again the severity of the phenotype was roughly related to the dosage of the abnormal genomes. This is consistent with clinical and biochemical findings in a family

in whom a mutation in mtDNA has been inferred because of a strong maternal inheritance pattern in the absence of major rearrangements.

3.4.4 Mitochondrial DNA and aging

Because of the relationships between senescence and the accumulation of abnormal mtDNA in fungi, and between energy metabolism and aging in man, it has been suggested that mtDNA may play a major role in aging in humans (Linnane et al.). Polarographic measurements on assymptomatic individuals undergoing orthopaedic surgery show a decline in enzyme activities over time (Trounce et al. 1989, Cardellach et al. 1989), and muscle histochemistry shows the occasional ragged red fibre or negative COX staining in the elderly (Mueller Hocker 1990). Although restriction mapping has not revealed any major re-arrangements in man, electron microscopic examination of mtDNA molecules which have been denatured and re-annealed suggests that point mutations accumulate with aging in rodents. Piko et al (1988) estimated that the proportion of mutated mtDNAs rose from 1% in adult to 5% in senescent rats. Similarly, point mutations have been shown to accumulate in the HeLa $tRNA^{thr}$ gene (Mita et al. 1988). Although still unproven, it is likely that this also occurs in man, but is not necessarily sufficient to explain the decline in respiratory activities described above. Mt RNA transcripts have also been shown to be lower in senescent than juvenile rats (Gadaleta et al. 1989), but the authors suggest that the mechanism for this may be related to alterations in metabolic requirements rather than mutations of mtDNA.

3.4.5 Mitochondrial DNA in carcinogenesis

Similarly, mtDNA re-arrangements have been discussed in the context of neoplasia (Shay and Werbin 1987). Both metabolic and morphological changes may occur in the mitochondria of neoplastic tissue, and some carcinogens can be shown to bind to mitochondria. It is known that an increased proportion of high molecular weight forms (dimers etc) have been documented in the blood of some patients with granulocytic anaemia (Clayton and Vinograd 1969), and accumulate in some cell lines (Howell et al. 1984). However, recent advances in the understanding of the mutations seen in tumours (reviewed Bradshaw and Prentice 1987) make it likely that mitochondrial changes are an epiphenomenum, and that the primary defect lies in mutations of nuclear DNA.

3.4.6 Mitochondrial abnormalities in other diseases.

There are many other conditions where mitochondrial mutations could be involved, but where mtDNA mutations have not been identified. The pattern of inheritance of congenital myotonic dystrophy might suggest a mutation in mtDNA (Harper 1979). However, it is clear that the simple hypothesis is inadequate; if abnormal mtDNA is also inherited by unaffected offspring it should also be transmitted to their offspring down the maternal line: there should therefore be individuals in the normal population who could mate with an affected male resulting in affected progeny with congenital onset. This has never been described, and it may well be that the pattern of inheritance of congenital myotonic dystrophy will be explained by imprinting.

There are a number of other conditions where the phenotype depends in some way on the parent from whon it is inherited, such as Huntington's chorea, neurofibromatosis and epilepsy. While the chromosomal location of the first two conditions have now been clarified by linkage analysis, none of the genes have not yet been cloned and so it is not yet possible to assess whether or not imprinting occurs. Epilepsy is likely to be multifactorial, and the increased recurrence risk in offspring of affected females over males may well be due to intra-uterine factors. However, myoclonic epilepsy occurs in the MERRF syndrome (section 3.3.1), it remains a tantalising possibility that the phenotype may be affected by an interaction of mitochondrial and nuclear genomes.

The biochemical defects found in Leigh's encephalopathy are quite variable, suggesting that this is a group of disorders rather than a single disease. Cytochrome oxidase deficiency has been described in a variable proportion of patients (DiRocco 1978), but so far generalised rather than specific deficiencies in mitochondrially encoded subunits have been documented (Miyabayashi et al. 1987). Complex 1 deficiency has also been documented (Robinson et al. 1987), as have morphologically abnormal mitochondria (Ohama et al. 1988). It seems likely that the causative mutation(s) will be nuclear rather than mitochondrial, possibly affecting enzyme assembly.

Schapira et al. (1989) have found complex 1 deficiency in brains from patients with Parkinson's disease. However, Bindoff et al. (1989) found defects in complexes 11 and 1V in addition to 1 in muscle form a similar group of patients. It is therefore possible that mitochondrial mutations may also be involved, although the suggestion that toxins are causative is more consistent with the sporadic occurrence.

In conclusion, mitochondrial mutations could be involved in a wider spectrum of disease than the three groups of conditions described in sections 3.4.1 to 3.4.3.

An investigation of the molecular genetics of some disorders of the mitochondrion.

Chapter 4: AIMS

The main aim of this project was to determine whether mutations of mitochondrial DNA (mtDNA) are responsible for certain diseases. The aims of the investigations described in each chapter were as follows:

Chapter 6: To investigate DNA from whole blood of 10 patients for the presence or absence of mitochondrial re-arrangements using five restriction enzymes and 11 regional probes.

Chapter 7: i To precisely map and sequence the duplications described in two patients

ii To begin to elucidate the role of these duplications in causing disease, by RNA analysis, and by investigation of the tissue distribution of deleted mitochondrial genomes.

Chapter 8: To investigate the possibility of detecting deletions in blood using the polymerase chain reaction (PCR), with a view to enabling non-invasive diagnosis of mitochondrial myopathy.

Chapter 9: To investigate patients with Leber's hereditary optic neuropathy (LHON) for the presence or absence of the mutation described by Wallace et al. 1988a, and identifying new mutations in patients lacking this mutation.

Chapter 5: Methods

NB Standard abbreviations for the usual reagents used in molecular biology will be assumed. These are defined in the glossary (Appendix 1), and where appropriate constituents are listed in section 5.10. All RNA reagents were made up in DEPC treated water (see section 5.10) using baked glasswear or RNAse free plastics, and autoclaved or filter-sterilised unless stated RNAase-free.

Enzymes were obtained from the following manufacturers: Amersham, New England Biolabs, Cetus, Pharmacia, Boehringer.

For all procedures the operator wore gloves (two pairs and laboratory coat for work with ^{32}P) and used sterile plasic ware. Most reagents were autoclaved or filter sterilised unless bought as DNAase-free, except for reagents used in electrophoresis, Southern blotting and hybridation of DNA.

5.1 Preparation of nucleic acids

5.1.1 Preparation of human genomic DNA from blood (after Old and Higgs 1983)

1. 2-20 ml of blood was obtained by venepuncture into up to 200 ul of 500mM EDTA in a 50ml falcon conical tube. This was centrifuged for 10 minutes at 3000 rpm (1,750g) at 4°C in a Beckman J6B centrifuge (rotor JS 4.2) and the plasma discarded, leaving the red cells and buffy coat. This was usually then stored at -20°C.

All volumes from here onwards are for 20ml of blood, but were scaled down for smaller volumes

2. After thawing the red cells were lysed by adding 30ml dH_2O and mixing. The tube was centrifuged for 10 minutes as before and the supernatant discarded to leave a white cell pellet.
3. The cells were then lysed by adding 25ml 0.1% Nonidet P40 and mixing. The contents were centrifuged as before and the supernatant discarded.
4. 10 ml of lysing solution (10mM Tris HCl pH 8.0, 10mM NaCl, 10mM EDTA) were added, followed by 0.5 mg of proteinase K (25 µl of a 20mg/ml stock solution in water) and 0.5ml of 10% sodium dodecyl sulphate (SDS). This was then left incubating for 4-18 hours, and if the original sample was clotted a second 25µl of proteinase K was added and left for 2 further hours.
5. This was extracted by adding 5ml Tris saturated phenol and 5ml of Chl/IAA. After mixing, this was centrifuged as before and the aqueous

supernatant carefully removed to leave any white proteinaceous material at the interface, which was discarded.

6. This was repeated, and two further extractions were performed in a similar way using 10ml Chl/IAA as above to remove the phenol.
7. DNA was precipitated by adding 1/10th volume of 4M NaCl and 2.5 volumes of absolute alcohol. This was left overnight at -20 °C or for 1 hour at -80°C, and then centrifuged as above for 15 minutes and the supernatant carfully removed to leave the DNA pellet.
8. The pellet was dried, left to dissolve in 1ml TE for 2-7 days at 4°C, and quantitated using a spectrophotometer. It is diluted X200 in water in order to estimate the yield and concentration: 1 OD unit at OD_{260} is 50ug/ml. The OD_{260}:OD_{280}gives a measure of the purity of the DNA and should be around 1.8.

5.1.2 Preparation of DNA from cultured white cells or bladder epithelial cell pellets. (after Hauswirth 1987)

1. The cell suspension was centrifuged for 10 minutes at 3000 rpm (1,750g) at 4°C in a Beckman J6B centrifuge (rotor JS 4.2) and the culture medium discarded, leaving a white cell pellet. These were either frozen in liquid nitrogen for storage at -80°C or extracted immediately
2. As steps 3 to 7 in 5.1.1
3. The DNA pellet was dissolved in 1ml of RNAase buffer (10mM HEPES pH 7, 50mM EDTA), 100 µg of DNAase free RNAase added (10µl of a 10mg/ml stock) and incubated at 37°C for 30 minutes.
4. This was then phenol/chloroform extracted as before (0.5 ml of each) and precipitated, dissolved in TE and quantitated as in steps 7 and 8 of 5.1.1

5.1.3 Preparation of human genomic DNA from frozen tissue. (H T Jacobs, Dept of Genetics, University of Glasgow, personal communication)

Two alternative methods were used, depending on whether or not RNA was also required for analysis (this required more tissue). The usual method, suitable for 3mg to 10g is described here.

1. The tissue was homogenised with a tissue homogeniser (Ultraturrax, Janke and Kunkel) at low speed in bursts of up to 60 seconds in at least 10 volumes of ice-cold lysis buffer (20mM Tris-Cl pH7.4, 100mM

NaCL, 10mM EDTA, 15mM EGTA). Proteinase K was added to 50µg/ml (or 250µg/ml for smaller tissue samples), and mixed well.

2. 20% N-lauroyl sarcosine was added to a final concentration of 1%, and incubated at 37°C for 30 minutes.
3. Protein was removed by phenol/Chloroform extraction: 1/2 volume of phenol and 1/2 volume of Chl/IAA were added, shaken for 1-2 minutes, and centrifuged at 3000rpm (1,750g) at 20°C for 10 minutes in a Beckman J6B with rotor JS 4.2, and the supernatant carefully removed.
4. Step 3 was repeated once or twice until the interface was clean. Residual phenol was then removed from the supernatant by two further extractions with 1 volume of Chl/IAA.
5. The DNA was precipitated by adding 1/10 volume of 3M sodium acetate pH 6.0, and 2.5 volumes of ethanol. This was left overnight at -20 °C or for 1 hour at -800C, and then centrifuged as above for 15 minutes and the supernatant carfully removed to leave the DNA pellet.
6. The pellet was dried, dissolved in a volume of RNAse buffer (as in step 3 5.1.2) which was about twice that of the original tissue.
7. RNAse was added to a final concentration of 50µg/ml and incubated at 37°C for 30 minutes.
8. Proteinase K was added as step 1, and then steps 2-5 repeated. The pellet was then dried and dissolved in TE (usually about 1/10 - 1/5 of the volume of the original tissue).

5.1.4 Preparation of human genomic DNA from hairs (Pilkerton et al. 1987)

This protocol is designed for use with hair roots from 20-30 freshly plucked hair roots. However, it was scaled down to 1/4 volumes for use with fresh single hairs. Insufficient DNA was obtained even for PCR when single shed hairs were used, even when stored at -20°C prior to extraction.

1. The hairs were cut up into 1-2mm lengths (generally just the 1cm nearest the root when several fresh hairs were available).
2. 200µl of lysing solution (5.1.1 step 4) were added, followed by SDS to 1.25%, dithiothreitol (DTT, to denature disulphide bonds) to 25mM, proteinase K to 2.5mg/ml (80µg of a 20mg/ml stock).
3. This was incubated overnight at 37°C.
4. It was phenol/chloroform extracted, using 1/2 volume of phenol and 1/2 volume Chl/IAA, and centrifuging for 5 minutes at 10,000rpm in a microcentrifuge.

5. The supernatant was extracted, using 1 volume Chl/IAA, and centrifuging for 5 minutes at 10,000rpm in a microcentrifuge.
6. The DNA was precipitated by adding 1/10 volume of 3M sodium acetate pH 6.0, and 2.5 volumes of ethanol. This was left overnight at -20 °C or for 1 hour at -80°C, and then centrifuged as above for 15 minutes and the supernatant carefully removed to leave the DNA pellet.
7. The pellet was dried and dissolved in 20-40µl of TE.

5.1.5 Large scale preparation of bacteriophage M13 DNA, replicative form from broth culture (after Maniatis et al. 1982)

1. Stationary phase E.coli were grown up overnight (usually strains JM101, JM109 or DH5αF') by innoculating a single colony from an M9 plate into 5ml of 2XTY.
2. A 1 in 100 dilution was made by adding 100µl of this to 10ml 2XTY, and innoculated with phage DNA by picking one plaque.
3. This was shaken at 37°C for 5 hours, then centrifuged in a bench microfuge for 5 minutes. This could be stored at 4°C for several months, or used immediately.
4. 1ml of the supernatant from 3 was added to a 1 in 100 dilution of stationary phase E.coli in 100ml of 2XTY, and shaken at 37°C for 5 hours.
5. The bacteria were pelleted by centrifugation at 3000rpm (1,750g) at 4°C for 10 minutes and the supernatant discarded.
6. They were resuspended in 2ml solution I (50mM glucose, 25mM Tris HCl (pH 8.0), 10mM EDTA, with 5mg/ml of lysozyme added just before use) and left at room temperature for 5 minutes.
7. 4ml of solution II (0.2M NaOH, 1% SDS) were added, shaking vigorously, and left on ice for 10 minutes.
8. 3ml of solution III (5M potassium acetate pH 4.8) were added, mixed and left on ice for 10 minutes.
9. This was transferred to a pre-cooled tube, and centrifuged at 20,000rpm (14,000g) in a Beckman J2-21 centrifuge with a JA20 rotor at 4°C for 20 minutes and the pellet discarded.
10. DNA was extracted from the supernatant by precipitation with 2 volumes of ethanol, and left at -20°C overnight.

11. This was centrifuged at 3000rpm (1,750g) in a Beckman J2-21 centrifuge with a JA 20 rotor at 4°C for 15 minutes and the supernatant discarded, the pellet dried and resuspended in 9.45 ml TE.
12. 9.45g of caesium chloride were dissolved in this, and 0.5ml of a ethidium bromide 10mg/ml and 0.5ml of dH_2O.
13. This was transferred to a Beckman 10ml polypropylene belltop ultracentrifuge tube, balanced to within 0.01g, sealed, and centrifuged at 36,000rpm (95,000g) for 60 hours at 18°C.
14. Bands were visualised under ultraviolet light corresponding to nicked plasmid (upper) and closed circular plasmid (lower). The lower band was pulled using a 1ml syringe after puncturing the top of the tube.
15. The ethidium bromide was removed by washing with one volume of isopropanol equilibrated with caesium chloride-saturated TE seven times, each time discarding the coloured layer (or with isopropanol saturated with sodium chloride three times).
16. The caesium chloride was removed by diluting four times with water, and precipitation with 10 volumes of ethanol.

5.1.6 Small scale preparation of bacteriophage M13 replicative form from broth culture (after Maniatis et al. 1982)

1. Stationary phase E.coli were grown up overnight (usually strains JM101, JM109 or DH5αF') by innoculating a single colony from an M9 plate into 5ml of 2XTY.
2. A 1 in 100 dilution of bacteria was made by diluting this with in 2XTY, and innoculated with phage by picking one plaque into 2ml.
3. This was shaken at 37°C for 5 hours
4. The bacteria were pelleted by centrifugation at 10,000rpm for 5 minutes and the supernatant discarded or used for preparation of ssDNA.
5. They were resuspended in 100µl of ice-cold solution I (50mM glucose, 25mM Tris HCl (pH 8.0), 10mM EDTA, with 5mg/ml of lysozyme added just before use) and left at room temperature for 5 minutes.
7. 200µl of solution II (0.2M NaOH, 1% SDS) were added, shaking vigorously, and left on ice for 5 minutes.
8. 150µl of solution III (5M potassium acetate pH 4.8) were added, mixed and left on ice for 10 minutes.
9. This was centrifuged at 10,000rpm in a microfuge for 10 minutes and the pellet discarded.

10. DNA was extracted from the supernatant by precipitation with 2 volumes of ethanol, and left at -20°C overnight.
11. This was centrifuged at 10,000rpm in a microfuge for 15 minutes and the supernatant discarded, the pellet dried and resuspended in 50µl TE.
12. This was reprecipitated where necessary by adding 1/10 volume of 3M sodium acetate pH6 and 2 volumes of ethanol and left at -20°C overnight.
13. This was centrifuged at 10,000rpm in a microfuge for 15 minutes and the supernatant discarded, the pellet dried and resuspended in 50µl TE.

5.1.7 Small scale preparation of phage ssDNA from broth culture (after Maniatis et al. 1982)

1. Steps 1 to 4 from 5.1.6 were carried out, and the pellet and remaining supernatant stored at 4°C. 1.25ml of supernatant was transferred to a clean eppendorf, recentrifuged at 10,000 rpm in a microcentrifuge for 5 minutes and decanted carefully to remove any residual bacteria.
2. 250µl of PEG/NaCl (20% polyethylene glycol, 2.5M NaCl) were added, mixed and left at room temperature for 15 minutes.
3. This was centrifuged at 10,000 rpm for 5 minutes and the supernatant completely removed (usually requiring another short spin) and discarded.
4. The pellet was resuspended in 200µl of TE, 100µl of phenol were added, vortexed 15 seconds, and left at room temperature for 15 minutes.
5. This was centrifuged at 10,000 rpm in a microcentrifuge for 3 minutes and the supernatant (approx 175µl) decanted carefully to a fresh tube.
6. The phenol was removed by extracting once or twice with Chl/IAA 200µl, mixing and centrifuging at 10,000 rpm in a microcentrifuge for 2 minutes and the supernatant decanted to a fresh tube.
7. The ssDNA was precipitated by adding 25µl of 3M sodium acetate pH6 and 600µl of ethanol and left at -20°C overnight.
8. This was centrifuged at 10,000rpm in a microfuge for 15 minutes and the supernatant discarded, the pellet dried and resuspended in 25µl TE.

5.1.8 Preparation of RNA with or without DNA from frozen tissue. (after Pharmacia data sheet 1988)

1. 2g of tissue (usually frozen in liquid nitrogen and stored at -80°C) was unfrozen, and sliced with a sterile scalpel. 8ml of GUT buffer was added (warmed to room temperature and filterd before use)

2. The tissue was homogenised using a tissue homogeniser (Ultraturrax, Janke and Kunkel) at low speed in bursts of up to 60 seconds
3. This was centrifuged at 1500rpm for 5 minutes and the supernatant divided in two and each was carefully added to 2.6 ml of 2g/ml CSFTA solution (10ml RNAase-free CSFTA was added to 7.83ml of 0.25M EDTA and 1.74 ml dH_2O) in a Beckman Polyallomer 14X89mm ultracentrifuge tube, so as not to disturb the interface.
4. Sterile mineral oil was added to within 1cm of the top of the tube, and they were then balanced to within 0.01g.
5. They were then ultracentrifuged at 31,000 rpm (120,000g) at 20°C for 16 hours.
6. The mineral oil and supernatant were then decanted and discarded.
7. The bottom 1/3 of the CSFTA layer was stored for DNA extraction (diluted X3 with dH_2O and preciptated with 2 1/2 volumes of ethanol, left at -20°C overnight and centrifuged at 3000rpm (1,750g) for 15 minutes at 4°C and the supernatant discarded, the pellet dried and dissolved in TE as in step 7-8 of 5.1.1)
8. The RNA pellet on the bottom of the tube was drained for 20 minutes by inverting the tube at room temperature. It was resuspended in 700µl of dH_2O and left on ice for 30 minutes. It was then precipitated by adding 70µl of 4M NaCl and 2 1/2 volumes of ethanol.
9. It was then left at -20°C overnight and centrifuged at 3000rpm (1,750g) for 15 minutes at 4°C and the supernatant discarded, the pellet dried and dissolved in 50µl of TE.
10. Quantitation was as for DNA (5.1.1 step 8) only 1/500 dilution was made. 1 OD unit corresponds to 40µg/ml.

5.2 Restriction enzyme analysis

5.2.1 Digestion of DNA

Type II restriction endonucleases were purchased commercially from Amersham International, New England Biolabs and Pharmacia for digestion of human total genomic, mitochondrial, PCR product and vector DNA. Restriction buffers were either supplied by the manufacturers, or made up according to their instructions, and stored in aliquots at -20°C. Reaction volumes were usually 20µl and comprised DNA, 10X buffer, enzyme (1-5u/µg of DNA) and water to 20µl. Reactions were performed in sterile micro-centrifuge tubes and incubated usually at 37°C for 1-16 hours. For Southern hybridisation of

genomic DNA, 1% minigels were run loading 1/10 of the sample to confirm complete digestion. A second aliquot of enzyme (half the first) was then added in some cases.

5.2.2 Southern Blotting

(Adapted from Southern 1975)

After the DNA had been digested, it was electrophoresed in 0.8-2.2% agarose gels (see section 5.3) and transferred to a nylon membrane by Southern blotting prior to hybridisation with a radiolabelled probe.

1. After staining and photographing the agarose gels, high molecular weight DNA was either fragmented by exposing to UV light for 60 seconds, or the gel was depurinated by immersing in 0.25M HCl for 15-30 minutes, if appropriate.
2. It was then denatured by immersing in denaturing solution (1.5M NaCl, 0.5M NaOH) for 45 minutes on a shaker, and then neutralised by immersing in neutralising solution (1M Tris-Cl pH7.5, 1.5M NaCl) for 45 minutes on a shaker.
3. It was then transferred to the blotting apparatus (a perspex board astride a tray containing 20XSSC, with a wick covering the perspex and dipping into the 20XSSC).
4. A nylon membrane (Hybond-N, Amersham) and two peices of Whatman filter paper were cut to size, soaked in 2XSSC and placed in that order on top of the gel. Bubbles were excluded by rolling a glass pipette over the pile.
5. The whole assembly was covered in Saran wrap, and a hole cut for the gel. Paper towels were placed on the top, with a weight to hold them down.
6. The blot was dismantled after 16-36 hours, marking the position of the wells prior to removal, the filter rinsed in 2XSSC dried at 37°C for 30 minutes and the DNA cross-linked to the filter by exposure to ultraviolet light for 90 seconds (on a Chromatovue transilluminator, model no TM20, UVP).
7. The gel could be restained with ethidium bromide (30 minutes in a solution of 500ng/ml) to check complete DNA transfer.
8. The filter could then be stored indefinitely at room temperature prior to use.

5.2.3 Hybridisation of DNA filters with radiolabelled DNA probes. (from Thein SL, Institute of molecular medicine, University of Oxford, personal communication)

1. The filter was prehybridised for 1/2 to 24 hours at 65°C in 5-10 ml of hybridisation solution (1.5XSSPE, 7.5% dextran sulphate, 100µg/ml sonicated salmon sperm DNA, 1%SDS, 5XDenhardts solution, 125µg/ml tRNA). This was performed by placing the filter in plasic bag cut to size and heat-sealed, carefully excluding all bubbles. Up to four filters were sucessfully hybridised in the same bag, increasing the volume of solution to ensure easy circulation over the filters.
2. dsDNA probes were denatured prior to introduction into the bag by boiling for 2 minutes (usually about 2 million counts per ml), and cooling on ice. A more uniform distribution was obtained if diluted in 1ml of hybridisation solution prior to introduction via a 1ml syringe and needle. The probe was distributed over the filters by spreading the solution gently back and forth.
3. This was left at 65°C overnight.
4. The stringency of post-hybridisation washes reqired depended on the properties of the probe itself, its concentration, specific activity etc, so after each wash each individual filter was checked with a hand-held geiger counter. Generally a 20 minute wash with a solution of 2XSSC, 0.1%SDS added at 65°C and cooling to room temperature on the shaker was sufficient for the regional probes. Radiolabelled purified mtDNA frequently reqired this to be repeated, followed by additional similar washes only using a solution of 0.2XSSC, 0.1%SDS added at 65°C.
5. Once the counts had fallen to <10 on the hand-held geiger counter, the filter was dried at 37°C for 30-60 minutes. It was then placed in an Xray casette with intensifying screens (first wrapping in saran wrap if not bone dry) with X-ray film (Fuji, RX)
6. Autoradiography was carried out at -80°C overnight, but for longer when necessary.
7. Filters could be re-used after washing off the probe with a 0.1%SDS solution at about 85°C cooling to room temperature for 30 minutes.

5.2.4 Hybridisation of DNA filters with radiolabelled oligonucleotides

(Ochman H, Department of Biochemistry, University of California, Berkely, USA, personal communication)

This was usually done with dot blots using one of two oligonucleotides corresponding to alternative sequences, but it was also used to probe Southern blots, and is listed here for that reason.

1. The filter was prehybridised as in 5.2.3 step 1, only using the following solution at 50°C: 6XNET, 0.5% NP40, 100µg/ml of nonhomologous DNA (such as E.coli or salmon sperm which was generally denatured prior to addition by placing in a micro-wave oven on full power for 2 minutes). Prehybridisation was at 50°C overnight.
2. The excess prehybridisation solution was squeezed out, and replaced with oligo-hybdridisation solution (6XNET, 0.5% NP40, 250µg/ml tRNA). End-labelled oligonucleotide was added in the same way as 5.2.4 step 2 at about 5,000,000 dpm/ml. Hybridisation was at room temperature overnight.
3. Post hybridisation washes were carried out as in 5.3.2 step 3, only using 6XSSC. 0.1% SDS. In the case of dot-blots the appropriate duration and temperature was ascertained using a test filter, and found to be 51°C for 30 minutes in a 51°C waterbath for ND4/W and 55°C for 30 minutes in a 55°C waterbath for ND4/L. For Southerns, the washes were much less stringent, using one or two washes of 5-15 minutes at room temperature.
4. Autoradiography was as in step 6 5.2.3.

5.3 Electrophoresis of nucleic acids

5.3.1 Agarose gels for DNA (after Maniatis 1982)

Horizontal agarose (Ultrapure, electrophoresis grade) gels were used for DNA analysis.

They were usually cast in TBE using International Biotechnology (Model HRH) gel boxes, and percentage varied from 0.8% to 2.2% for detection of large and small fragments respectively. Agarose was dissolved in buffer by heating in a microwave oven. Ethidium bromide staining to visualise the DNA was either carried out by adding 0.5µg/ml to the agarose and running buffer, or by incubating the gel in a 1µg/ml solution for 30 minutes at room temperature after electrophoresis (in some cases subsequent de-staining in water for 30-60 minutes was also necessary). Voltages were varied from 1 to 5V/cm

depending on gel composition and fragment sizes, using a Biorad (model 200/2.0) power pack.

Low melting point agarose in TAE was generally used for preparative gels. Percentages ranged from 0.6 to 1.0%, and voltages 1-1.5 V/cm.

Size markers were used in all cases, usually a digest of bacteriophage lambda with Hind III or the 1kb markers (both BRL). Loading buffer (6X buffer: 0.25% bromophenol blue, 0.25% xylene cyanol, 15% Ficoll (type 400) in dH_2O) was added to samples prior to loading.

Ethidium bromide stained fragments were visualised on a UV transilluminator (Chromatovue, model no TM20, UVP) and photographed.

5.3.2 Polyacrylamide gels for sequencing (after Amersham M13 cloning and sequencing handbook 1984)

Polyacrylamide gels were poured between glass plates using the sliding plate method. Gel composition varied from, 6-8% depending on the distance to be read from the primers. For a typical 6% 60ml gel, 100ml of the following were prepared: 42g urea (ultrapure)

15ml acrylamide stock solution (40% solution of 19:1 acrylamide: NN'-methylenebisacrylamide, store 4°C)

10ml of 10X TBE buffer

dH_2O to 100ml

The urea was dissolved at 37°C, cooled to room temperature, and polymerised by adding 1ml 10% solution of ammonium persulphate and 15µl TEMED (NNN'N'-tetramethylethylenediamine) just before use.

Samples were loaded in "stop solution " (or loading buffer) after heating to 75-90 °C for Voltages and running times varied from 400 to 1500V and 2-24 hours respectively, using a BRL S2 sequencing gel box and Biorad 3000Xi power pack. Where long lengths of sequence were to be read, either long runs were to be performed (running buffer was changed up to 3hourly) or buffer gradient gels were used (by adding 1M potassium acetate to the bottom reservoir).

Gels were then dried for 45 minutes at 80°C using a Biorad 583 gel drier, and the saran wrap removed prior to autoradiography at room temperature for 1-7 days.

5.3.3 Denaturing gels for RNA analysis

1.2-1.4% agarose gels were cast in a gel box as above. A typical 1.2% gel contained: 1.2g agarose

10ml 10X MOPS buffer (0.2M morpholinopropranesulphomic acid, 0.05M anhydrous sodium acetate, 0.01 M disodium EDTA, pH7.0)

75 ml dH_2O

The agarose was dissolved by heating in a microwave oven, and 15ml of formaldehyde (37%) added prior to pouring.

The running buffer was 1X MOPS buffer, and the samples were loaded in a solution containing 50% de-ionised formamide, 1X MOPS buffer, 15% formaldehyde and 0.05% bromophenol blue, and heated to 55°C for 5 minutes before loading.

Gels were run with RNA size markers (BRL) at 20-60 V (0.7-1.5 V/cm)

RNA was visualised by staining in a 3µg/ml solution of acridine orange in MOPS buffer for 30 minutes at room temperature, and then de-stained in MOPS buffer for 20 minutes. It was then illuminated using a UV transilluminator (Chromatovue, model no TM20, UVP) and photographed.

5.4 Radiolabelling of nucleic acids

5.4.1 Random hexanucleotide priming of dsDNA and ssDNA probes (Feinberg and Vogelstein 1983).

1. This was carried out using the Amersham Multiprime labelling kit. Starting templates were either 2ng of purified total mtDNA or 12.5ng of regional probe in M13 both linearised by digestion with BamH 1, or occasionally gel-purified PCR product or probe insert excised by digesting with Eco Rl and Hind lll.
2. The DNA was denatured by boiling for 2 minutes, and then plunging on to ice. For excised bands in low melting point agarose, the DNA was denatured by heating to 95°C for 10 minutes and then plunged into a 37°C waterbath (in the latter case all other reagents were mixed and then added to the DNA).
3. The following reaction was set up in a volume of 25µl (adding the water

first and the enzyme last):

DNA	
5Xbuffer	5.0µl
Primer/BSA	2.5µl
α^{32}P-dCTP	2.5µl
Klenov fragment of DNA polymerase I	1.0µl
Water to 25µl	

4. The reaction was left at 37°C for 30 minutes.
5. The probe could either be used immediately, or separated from unincorporated α^{32}P-dCTP by putting it down a sephadex column and the incorporation measured (sephadex G50 was mixed with 4 volumes of dH_2O, autoclaved and cooled overnight. A column was constructed inside a 1ml syringe by plugging the bottom with siliconised glass wool, and packing by centrifuging at 2000rpm (450g) in a centaur benchtop centrifuge for 4 minutes, and equilibrated by repeating this centrifugation until 100µl of dH_2O placed on the top before the spin came out of the bottom. The probe was then diluted to 100µl and centrifuged for 4 minutes).
6. Percentage incorporation could be calculated by placing equal proportions of the whole from before and after purification in a scintillation counter. Actual dpm was calculated from the known specific activity of the starting isotope. It was usually around 60%.

5.4.2 Kinasing oligonucleotides with gamma ^{32}P ATP (from Higuchi R, Cetus corporation, Berkely, USA, personal communication)

1. 40pmol of oligonucleotide was labelled in a 20µl reaction volume. The following reagents were added:

Oligonucleotide	
10Xkinase buffer	2µl
gamma ^{32}P ATP	3µl
T4 polynucleotide kinase (Amersham)	0.5µl ie 1.5units
dH_2O to 20 µl	

2. Incorporation was maximum for a 15-20 minute incubation at 37°C, after which it fell.
3. The probe could then be used immediately, stored at -20°C after inactivating the kinase by heating to 95°C for 10 minutes, or separated from unincorporated gamma ^{32}P ATP using a spun column (5.4.1 steps 5 and 6 only using sephadex G50).

5.5 Polymerase Chain Reaction (PCR)

5.5.1 PCR Protocol (Erlich 1989)

1. Thermostable Taq polymerase (Cetus corporation) was used (1unit per 50µl reaction volume) with buffers of various concentrations of $MgCl_2$ (1.0 to 4.0 mM) and 200µM of each dNTP, and a computer controlled alternating temperature water bath (courtesy of Dr Tom Kocher). The most frequently used 1Xbuffer was 50mM KCl, 10mM Tris-Cl pH 8.3, 1,5mM $MgCl_2$, 0.01% gelatin (gelatin may be omitted)
2. Denaturation was at 93°C for 1 minute, annealing at 37°C to 62°C for 1 to 2 minutes and extension at 73°C for 1 to 5 minutes for 10 to 40 cycles.
3. Optimum conditions were established for each primer pair where cloning or sequencing was to be performed (ie concentration of $MgCl_2$, primers, annealing temperature, extension time, see table 5.3)
4. Number of cycles required depended on aims of individual experiments, and starting concentrations of template. In general, where pure product yield was to be maximised, PCR was terminated near the start of the plateau phase.
5. Starting quantities of DNA depended on the purpose of the PCR. a) For a single round of PCR, 100ng of total genomic DNA or 2ng of purified mtDNA was usually sufficient. Although it was noted that yields could be increased by prior linearisation usually with Bam HI, there was a theoretical objection which disallowed it: a new restriction site might be present in a mutant population of mtDNA, so that the yield from PCR of the mutant might be considerably reduced by prior digestion.
   b) Where a second round of PCR was performed, 1/25 of the first product was taken (more gave higher backgrounds) except for COP.
6. A typical 50µl reaction would comprise the following:

100ng template DNA	2.0µl
Primer A (10µM)	1.5µl
Primer B (10µM)	1.5µl
10Xreaction buffer	5.0µl
dNTP solution (40mM)	1.0µl
Taq polymerase 1unit	2.0µl

with dH_2O to 50µl and sterile mineral oil to cover (approx 50µl)

5.5.2 PCR primers

Primers were designed to be complementary to unique mitochondrial DNA sequences, and to minimise possible secondary structure which might prevent efficient PCR. The criteria used are listed below, and the primer positions are illustrated in fig 5.2 and listed in table 5.2. The optimised conditions for various primer pairs are listed in table 5.3. Most were synthesized and HPLC purified commercially

1. Length was usually 20bp, as this provided sufficient specificity, without too high a chance of a mismatch as a result of a point mutation within the template sequence corresponding to the primer. In the case of competitive oligonucleotide priming (COP, described below) 16mers were used as longer primers may tolerate one or more mismatches.
2. Possible primer sequences were compared to the reference sequence (Anderson et al. 1981) using the Staden package and the EMBL database, and rejected where highly homologous to other regions of mtDNA (in practice matches in excess of 13 or 14bp were usually avoided, especially when these occurred at the 3' end).
3. Where possible, primers were designed to be complementary to highly conserved sequences, either in a tRNA or 3' end to end on a first or second position in a codon.
4. Extremes of base composition were avoided (usually between 3 and 7 of any one base in a 20mer).
5. Tm between 56 and 68 °C
6. Homopolymeric runs in excess of three were avoided.
7. Inverted repeats were avoided by inspection.

5.5.3 PCR with crude lysates (Saiki et al. 1986)

1. To 10µl of cells in culture medium (or 2µl of blood diluted with PBS to 10µl) was added 35µl of dH_2O.
2. This was heated to 95°C for 10 minutes, cooled to 65°C, and the remainder of the PCR "master mix" added before going on to the annealing stage of the first cycle.

5.5.4 PCR for direct sequencing of single stranded PCR products (after Gyllensten 1989)

1. 30 cycles of PCR were performed, the product reduced to 10µl volume in a speedvac, and electrophoresed in a 1% TAE minigel.

2. The gel was stained with ethidium bromide, the band cut out and eluted into TE at 4°C overnight. This was again subject to PCR, only this time the primers were added in a ratio of 100:1 (ie one at 1μM and the other at 10nM) and 40 cycles were performed.
3. The product was then dialysed in TE by 3 cycles of centrifugation in a centricon 30 (Amicon) at 5000rpm (3000g) in a Beckman J2-21 centrifuge using a JA 20 fixed angle rotor at 20°C, and collected in 30-40μl by inverting and centrifuging at 1500rpm (340g) in a Beckman J6B with a JS4.2 rotor for 5 minutes.

5.5.5 PCR and contamination

Contamination was excluded by the following precautions

1. All buffers and non-sterile plastic-ware were autoclaved before use.
2. Ultrapure water and mineral oil were obtained commercially.
3. All stock reagents (buffer, dNTPs, oligonucleotides both working solutions and concentrated stocks, water and mineral oil) were stored in aliqots.
4. Individual reactions were generally set up from a PCR "master mix" containing everything except DNA, so that simultaneous PCR was performed on controls, unaffected relatives other patients and water blanks using identical reagents.
5. DNA solutions containing high concentrations of the target sequence such as plasmids and PCR products were rarely used as controls.
6. Nested PCR minimises problems with contamination. The product of the final round of PCR will not be amplified if it contaminates the samples about to undergo their first round of PCR as it is too short, and the product of the first round is not usually present in a high concentration in any tube.

5.5.6 Competitive oligonucleotide priming (COP) (Gibbs et al. 1989)

1. Alternative primers corresponding to wild and mutant sequences were designed to be complementary to a 16bp sequence containing the region of the point mutation (primers ND4/W and ND4/L respectively).
2. ND4/W and ND4/L were kinased with gamma ^{32}P-ATP as in section 5.4.2.
3. PCR products were prepared from patients' blood DNA using primers ND4/6 and ND4/7. These were then used as the template in the COP

reaction. A typical 50µl tube contained 100ng ND4/6-ND4/7 template, 500nM of each of primers ND4/6, ND4/W and ND4/L, 4mM $MgCl_2$, remaining reagents as in 5.4.1. For each patient two such identical reactions were performed, except that they were "spiked" with either labelled ND4/W or ND4/L (approx 20nM).

4. Non-specific priming was avoided using the following cycle: Denature 93°C 1 minute, anneal 48°C 2 minutes, extend 55°C 1 minute, for 20 cycles.
5. 1/10 of the product was loaded on to a 2% agarose gel, run at 7V/cm, and dried at 50°C for 45 minutes then 80°C for 15 minutes using a Biorad 583 gel drier, and covered in saran wrap prior to autoradiography at -80°C with intensifying screens for 1-3 days.

5.6 analysis of PCR products: oligonucleotide probing (from Wordsworth P, institute of molecular medicine, University of Oxford, personal communication).

1. Roughly equal quantities of PCR product (100-200ng) were diluted to 100µl with TE. 6µl of 0.5M EDTA and 8 µl of 6M NaOH were added to each to denature the DNA, and left on ice for 10 minutes. 110µl of 2M ammonium acetate were added, and the samples loaded on to the dot-blot apparatus (This had been prepared by soaking a nitrocellulose filter and two layers of Whatman paper, cut to size, in dH_2O, and mounted in the apparatus which had been connected to the water-controlled vacuum line. 200µl 1M ammonium acetate had been loaded into each well just prior to loading the samples).
2. 200µl of ammonium acetate was loaded into each well just after loading, and the apparatus unloaded and the filter dried and baked (2hours at 80°C in a vacuum oven, which also fixes the DNA.)
3. Hybridisation with end-labelled oligonucleotide.was similar to that described in section 5.2.4.

5.7 analysis of PCR products: cloning.

5.7.1 Vectors

Strategy I: (from Bell JI, Institute of Molecular Medicine, University of Oxford, personal communication). Because of the low efficiency of blunt-ended ligations, PCR products were initially cloned into a modified bacteriophage M13 mp8 (Way et al.) with an EcoK site flanking an EcoRV

cloning site inserted in the polylinker. Theoretically this cut down the proportion of white plaques when used in conjunction with an Ecoli strain such as JM101 in which the restriction system would recognise an intact EcoK site in a religated vector containing no insert, and digest it. Unfortunately, because of nonspecific DNAse activity in the EcoRV, a high proportion of white plaques contained no insert but had lost one or two bases by this means resulting in a frame shift.

Strategy 11: (Robson K, Institute of Molecular Medicine, University of Oxford, personal communication). The vector was therefore replaced by a commercially obtained SmaI cut bacteriophage M13 mp8 (Amersham), phosphatased to prevent re-ligation of vector alone. Although this involved further manipulation of the PCR product in order to kinase the ends (see below), the background was much lower. Furthermore, it was possible to use other strains of E coli, such as DH5αF', which gave generally higher cloning efficiencies.

5.7.2 Preparation of insert and ligation

Strategy 1: PCR products were separated from unincorporated dNTPs using a Centricon separator. Any ragged ends of the PCR products were filled in with the Klenow fragment of DNA polymerase 1, by incubating in a 10ul reaction volume with 5U enzyme at 37 °C for 15 minutes in 10mM Tris HCl pH 7.5, 5mM $MgCl_2$ 1mM DTT, 0.05mM each dNTP. This was then extracted once with phenol, 4 times with chloroform/IAA and twice with ether. These were then cloned into a modified bacteriophage M13 mp8 using standard conditions for cDNA cloning. Each ligation reaction of 10µl contained 20ng of vector, and 500pg to 60ng of insert (from 1:0.5 to 1:20) in 100mM Tris-HCl pH 7.5, 10mM $MgCl_2$, 20mM DTT, 1mM rATP, 50pg/µl bovine serum albumen, with 200 units of New England Biolabs ligase at 16°C overnight.

Strategy 11: PCR products were gel purified using agarose gels in acetate buffer. The products were eluted into TE overnight at 4° C and any ragged ends of the PCR products were filled in with the Klenow fragment of DNA polymerase 1, as above and then separated from unincorporated dNTPs using a Centricon separator, kinased (using Amersham polynucleotide kinase 1.5 units in Tris-HCl pH 8 50mM, $MgCl_2$ 7mM, DTT 1mM at 37°C for 20 minutes, then inactivating the kinase by heating to 65°C for 10 minutes) and cloned into SmaI cut phosphatased bacteriophage M13 mp8 (Amersham) using standard conditions for cDNA cloning as above.

5.7.3 Transformation

Strategy 1: The ligated product was then used to transform E.coli JM101 (Raleigh et al. 1988) using the Hanahan (1985) protocol exactly as described Strategy 11: The ligated product was then used to transform E.coli DH5αF' (Raleigh et al. 1988) using the Hanahan (1985) protocol. This was modified in only one respect: the initial culture taken from an M9 plate was grown up overnight in SOB broth rather than on a plate. The next day a 1 in 100 dilution in SOB was made as this gave a more reproducible bacterial load than picking colonies from an SOB plate. This was grown up at 37°C in a shaking incubator for 2h 45 minutes, rather than measuring sequential optical densities which slowed growth significantly. The rest of the Hanahan (1985) protocol was followed exactly as described. This modification did not impair transformation efficiencies which were generally 10^7 or 10^8 per μg vector DNA.

5.7.4 Screening of clones

White plaques were picked and grown up using standard methods. They were screened by crude extraction of ssDNA using EndoRstop buffer (Robson K, Institute of Molecular Medicine, University of Oxford, personal communication), and electrophoresed to detect large inserts. ssDNA was prepared (as described in section 5.1.7) from probable recombinants, and sequenced (5.8.1)

5.8 analysis of PCR products sequencing:

5.8.1 Single stranded DNA sequencing.

DNA was sequenced by the dideoxy method using the Sequenase (USB) kit with ^{35}S-dATP and either the universal m13 primer or sequencing primers as listed above, exactly as in the manufacturer's instructions. The template used was either clones in M13 (see section 5.1.7) or single stranded PCR products (section 5.5.4). Sequence was analysed using the Staden package and the EMBL database.

5.8.2 Direct sequencing of double-stranded PCR products. (Brown J, Institute of Molecular Medicine, University of Oxford, personal communication)

DNA was sequenced by the dideoxy method using the Sequenase (USB) kit with ^{35}S-dATP. 50-125ng of gel-purified template were sufficient.

1. The template was denatured with 1pmol primer in sequenase buffer at 95°C for 7 minutes, then annealed rapidly in a dry ice/ethanol bath for 2 minutes.
2. To the above (10µl) was added:

0.1M DTT	1.0µl
kit dGTP mix (diluted 1 in 10)	2.0µl
^{35}S-dATP	0.3µl
Sequenase (diluted 1 in 8)	2.0µl

   and incubated on ice for 3 minutes
3. 4 tubes were set up for each reaction, using the dideoxy/deoxy mixes from the kit. 3.5µl of reaction in step 2 was added to each tube and incubated at 37°C for 3 minutes.
4. The reaction was terminated by adding 4 µl of loading buffer, and loaded as above.

5.9 RNA analysis

5.9.1 Northern analysis:

RNA extraction is described in section 5.1.8, and electrophoresis followed by staining and photographing the gel in section 5.3.3. The gel was then incubated in 20XSSPE for 20 minutes, and a blot set up as described for DNA in section 5.2.2, only using 20XSSPE instead of SSC. The filter was dried and the RNA cross-linked to the filter by exposure to ultraviolet light for 90 seconds (on a Chromatovue transilluminator, model no TM20, UVP).

5.9.2 Hybridisation of RNA filters with radiolabelled oligonucleotides.

(from Amersham Membrane transfer and detection methods, 1985)

1. This procedure was almost identical with that in 5.2.4 steps 1 and 2, only basic prehybridisation and hybridisation solutions were re-sterilised prior to use, usually by filtering.
2. Post hybridisation washes were carried out as in 5.3.4 step 3 using 6XSSC. 0.1% SDS warmed to 37°C for 30 minutes in a 37°C waterbath.
3. Autoradiography was as in step 6 5.2.3.

5.9.3 cDNA synthesis (from Dallman M, Nuffield department of Surgery, University of Oxford, personal communication).

1. Approximately 12µg of RNA in TE was DNAase treated in a 50µl reaction volume containing 1µl of RNAase-free Bovine pancreatic DNAse 23 units

(Boehringer), 5mM $MgCl_2$, 1µl of human placental RNAase inhibitor, 60 units (Boehringer) at 37°C for 30 minutes. It was then phenol extracted once, chloroform extracted once and ether extracted twice.

2. One third of this (4µg) was annealed to 0.2 ODU of oligo dT (Pharmacia) in a 24µl volume by heating to 60°C for 5 minutes, and then plunging on to ice for 3 minutes.

3. For the reverse transcriptase reaction, the following reagents were added: 1µl of human placental RNAase inhibitor, 60 units (Boehringer), 4µl of the dNTP stock used in PCR (10mM each dNTP), 8µl of 5X reverse transcriptase buffer (supplied with enzyme) and 2µl of murine-maloney virus reverse transcriptase 400 units (BRL). This was incubated at 37°C for 40 minutes, then 2 µl more enzyme were added and incubated again at 37°C for 40 minutes.

4. This was made up to 100µl with dH_2O, was then phenol extracted once (back extracting the phenol once), chloroform extracted twice and precipitated by adding 3M sodium acetate pH5.2 (to a final concentration of 2M) and with 2.2 volumes of ethanol.

5. This was left overnight at -20 °C or for 1 hour at -80°C, and then centrifuged as above for 15 minutes and the supernatant carefully removed to leave the DNA pellet.

6. The pellet was then dried and left to dissolve in 100µl water. 5µl of the resulting cDNA was then used as a template in PCR reactions designed to detect specific RNAs.

5.10 Stock Reagents (if not already covered in text)

Chl/IAA 24:1 mixture of chloroform and isoamyl alcohol

DEPC: diethylpyrocarbonate, added to dH_2O at a dilution of 1 in 1,000 and autoclaved

50XDenhardts: 5g Ficoll (type 400 Pharmacia), 5g polyvinylpyrrolidine, 5g bovine serum albumen, in water to 500 ml and filtered.

EDTA: 0.5M solution of disodium ethylenediaminetetra-acetate pH 8.0 with NaOH

GUT buffer: 4M guanidinium isothiocyanate, 0.5% sarcosyl, 25mM Na citrate pH 7.0. Store at 4°C, filter before use and add RNAse-free ßME to 0.1M and RNAse-free antifoam to 0.1%.

dH_2O: De-ionised water

H top agar: per litre: 10g bacto tryptone, 8g NaCl, 8g agar.

M9 plates: the following were autoclaved seperately and cooled befor mixing: 15g minimal agar in 900ml of dH_2O, 100ml 10X M9 salte (per litre 60g Na_2HPO_4, 30g KH_2PO_4, 10g NH_4Cl, 5g NaCl), 1ml 1 M $MgSO_4$, 1ml 0.1 M $CaCl_2$, 1ml 1M thiamine HCL, 10ml 20% glucose.

MOPS: 3-[N-Morpholono]propanesulphonic acid

PBS: NaCl 120mmol, KCL 2.7 mmol in phosphate buffer (Na_2HPO_4, KH_2PO_4 pH 7.6 (Sigma).

Phenol: prepared by equilibrating first with 1M tris chloride pH 8 then 0.1M tris pH 8

TAE: Tris-acetate-EDTA electophoresis buffer: 40mM Tris acetate, 1mM EDTA

TBE: Tris-borate-EDTA electophoresis buffer: 89 mM Tris-borate, 89 mM boric acid, 2.5mM EDTA pH8.3.

TE: 10mM Tris HCl, 1mM EDTA, pH 7.5

2XTY medium: per litre, 16g bactotryptone, 10g yeast extract, 5g NaCl

2XTY plates: add 15g/litre bacto agar to 2XTY medium

2XTY plates with ampicillin: add 30-50mg ampicillin to 1 litre of 2XTY agar as above

20XSSC:175g NaCl, 88.2g sodium citrate to pH 7.0 with NaOH

SSPE: per litre: 175.3g of NaCl, 27.6g $NaH_2PO_4.H_2O$, 7.4g EDTA with NaOH to pH7.4.

Table 5.1 Probe positions and sizes (according to King M, University of Columbia, New York, personal communication, who kindly supplied the probes) relative to the reference sequence (Anderson et al. 1981).

Probe number	Position		Length (bp)	Light or Heavy strand
1	16,134	41	476	H
2	41	2,578	2,537	L
3	2,578	4,122	1,544	H
4	4,122	5,374	1,153	H
5	5,275	6,204	927	L
6	6,204	7,441	1,237	H
7	7,441	8,287	846	L
8	8,287	8,592	305	H
9	8,592	9,648	1,056	H
10	8,729	10,254	1,525	H
11	10,254	11,922	1,668	L
12	11,680	12,570	890	H
13	14,956	16,053	1,097	H
14	15,591	16,569	979	H

Table 5.2

Positions of oligonucleotide primers relative to the reference sequence (Anderson et al. 1981).

NAME	LIGHT OR HEAVY STRAND	BASE NO from	to
tRNA Y	L	5,864	5,883
CO1/1	L	6,004	6,013
CO1/2	L	7,414	7,433
CO1/3	L	7,193	7,212
CO1/4	H	7,380	7,399
A	H	8,297	8,316 =
B	L	8,196	8,215 =
C	L	8,225	8,244 =
CO111/1	L	9,976	9,995
ND4/1	L	11,090	11,109$
ND4/2	H	11,395	11,415$
ND4/3	H	12,172	12,191
ND4/4	L	11,211	11,230&
ND4/5	H	11,139	11,158
ND4/6	L	11,632	11,651
ND4/7	H	11,843	11,862
ND4/W	H	11,769	11,784
ND4/L	H*	11,769	11,784
Hinc11/1	L	13,209	13,232 @
Hinc11/2	H	13,286	13,305 @
ND5/1	H	13,812	13,831
ND5/2	L	13,707	13,726
Cytb1	H	14,974	14,955
Cytb2	L	14,900	14,919
Cytb3	H	15,132	15,151
tRNAt	H	15,938	15,957
PRO	L	16,000	16,019$
D3	H	16,259	16,278+
D5	H	16,401	16,420+
D8	L	110	129+
D10	H	325	344+
PHE	H	580	599$
SA junction oligo	H#	6,121	6,130]
		15,056	15,065]

* Corresponds to heavy strand sequence except for base pair 11,778 (see Chapter 9)
# See chapter 7
$ From Stoneking M, University of Californina, Berkely, California, USA
+ From Kocher T, University of Californina, Berkely, California, USA
& From Vigilant L, University of Californina, Berkely, California, USA
= From Wrischnik et al. 1987
@ From Paabo et al. 1988

Table 5.3

Conditions used for various combinations of primers in the polymerase chain reaction

L strand primer	Heavy strand primer	volume of stock solution (1.2 ODU/ml or 10 µM) to a 50µl reaction in µl	Magnesium Conc (mM)
B	ND5/1	1.5	1.5
tRNA Y	Cytb3	1.5	1.0
CO1/3	tRNA T	0.5	3.0
tRNA Y	tRNA T	0.5	1.0
ND4/6	ND4/7	0.1	1.0
CO1/2	Cytb3	1.5	1.5
HincII/1	ND5/1	1.5	2.0
CO1/3	A	0.5	4.0
ND5/2	Cytb1	1.5	2.0
B	Cytb3	1.5	1.5

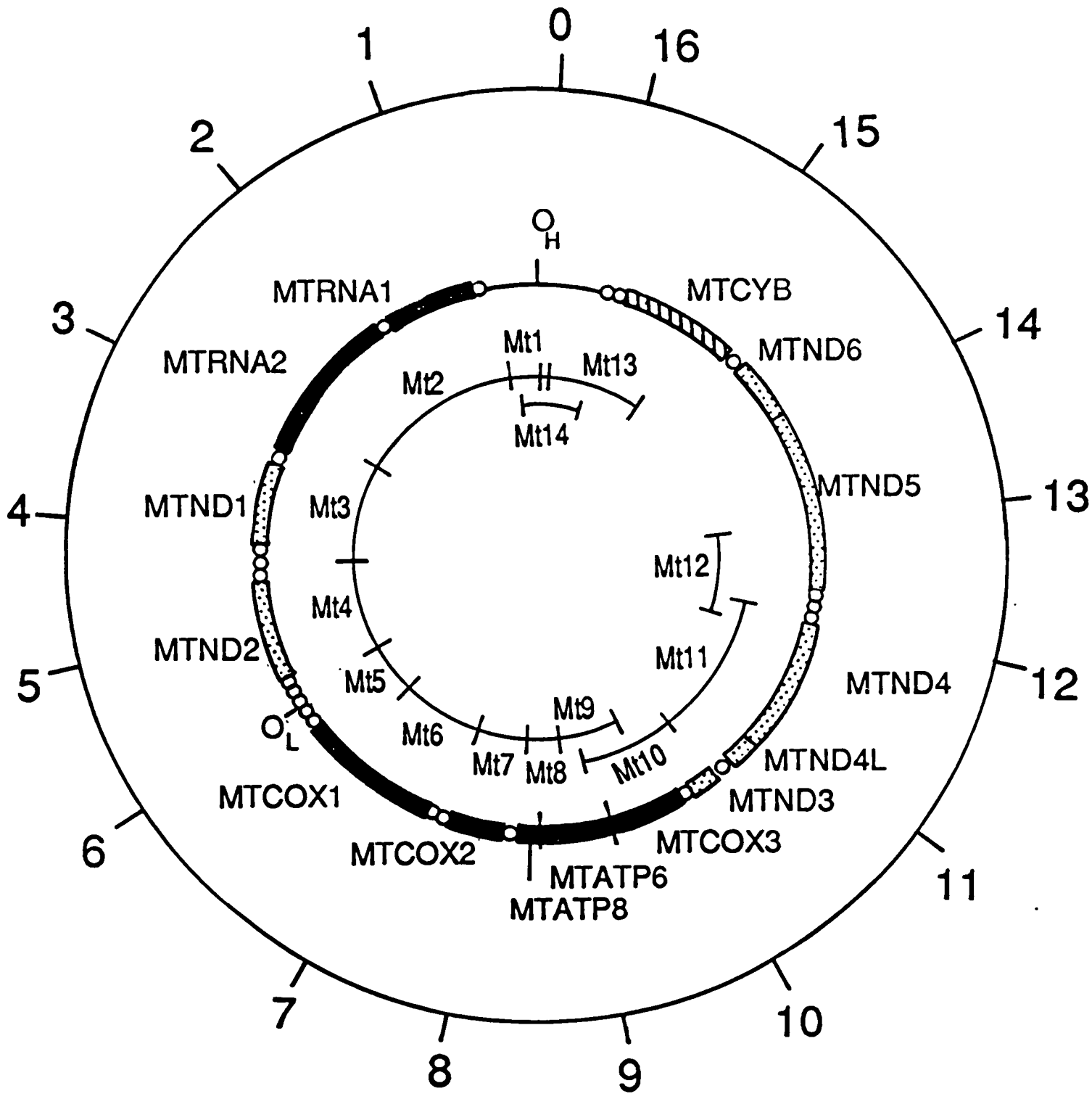


Figure 5.1

The human mitochondrial genome showing location of probes used. MTCOX1 to MTCOX3: subunits 1 to 3 of cytochrome C oxidase. MTND1 to MTND6, and MTND4LL subunits 1 to 6 and 4L of NADH-dehydrogenase. MTATP6 and MTATP8: subunits 6 and 8 of H^+-ATPase. MTCYB: apocytochrome B. MTRNA1 and MTRNA2: 12s and 16s rRNA subunits. MT1-14: regions corresponding to probes 1 to 14 referred to in text. 0: transfer RNAs.

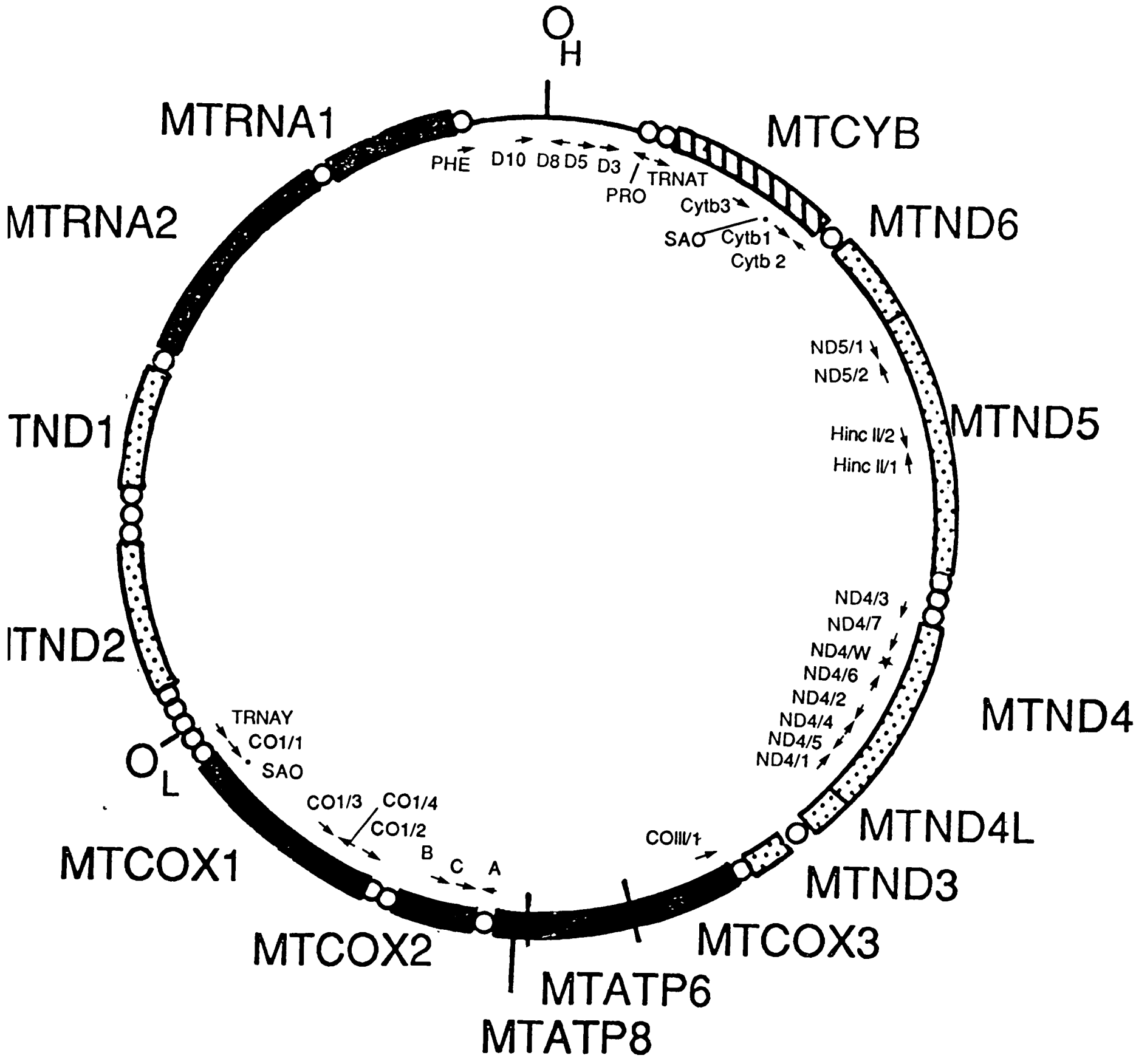


Figure 5.2 Positions of 20mer oligonucleotides used (see table 5.2)
Arrows represent directions of primers but are not drawn to scale
: position of junction oligonucleotide used in patient 1 (both halves marked with a seperate dot)
: position of 16mer oligonucleotide used in LHON patients, complementary either to wild-type or mutated sequence at base no 11,778.

Chapter 6: Results

Restriction enzyme analysis of blood mtDNA in patients with mitochondrial myopathy

NB: Please refer to appendix 1 to cross reference patients appearing both in this chapter and chapter 8.

6.1 Summary

Restriction enzyme analysis of mitochondrial DNA from blood of 10 patients with mitochondrial myopathy using five enzymes did not reveal any major re-arrangements in 83% of the protein coding regions. Similarly, the only point mutations in the 5% of this region which comprised the restriction sites were probably population polymorphisms.

6.2 Introduction

Although the association between mutations of mitochondrial DNA (mtDNA) and mitochondrial myopathy (MM) is now well established, little was known at the time that this study was initiated. The hypothesis that mutations of mtDNA might be associated with MM was reasonable both because of the predominantly maternal inheritance pattern in familial cases (Egger and Wilson 1983, Rosing et al 1985) and because of the association with biochemical defects of the electron transport chain (Petty et al.1986).

As muscle biopsy material was not readily available to us, it was decided to perform a preliminary investigation on blood. Although heteroplasmy was known to be a theoretical possibility, available data on normals and leukaemic patients suggested that homoplasmy was the norm. Furthermore, in the most extreme case of heteroplasmy which had been described in a related species, the variation in genotypic ratios different tissues only varied from 2.2:1 to 3.3:1 (Hauswirth and Laipis 1985). It was therefore reasonable to do an initial study on mtDNA in blood. Because preliminary work by our collaborator, Dr Atul Mehta, had not revealed any abnormalities using purified total mtDNA as a probe (Mehta 1987), it was decided that this should be supplemented by the use of regional human mtDNA probes in bacteriophage M13 (fig 5.1, courtesy of Professor Attardi and Dr M King). These were able to give more detailed unambiguous regional information.

Table 6.1

Clinical details of patients, including muscle data which became available afterwards.

Patient no.	Sex	FH	Biochemical defect	Deletion PCR on blood	Muscle blot	Category
1[1]	M	-	I	No	NA	CPEO
2[2]	M	-	NA	No	No	CPEO
3[3]	M	*	-	Yes (cd)	Yes	KSS
4[4]	M	-	I & IV	Yes	Yes	CPEO
5[2]	F	-	-	Yes (cd)	Yes	CPEO
6[2]	F	+	I	No	No	II
7[2]	F	-	NA	No	NA	MELAS
8[2]	F	-	I	No	No	MELAS
9[5]	M	-	V & CN	No	NA	MERRF
10[2]	F	-	III & IV	No	No	MERRF

[1] see Land et al. (1981), [2] see Poulton et al. (1988),[3] see Holt (1989), [4] see Johnson et al. (1983), [5] see Petty et al. (1986)

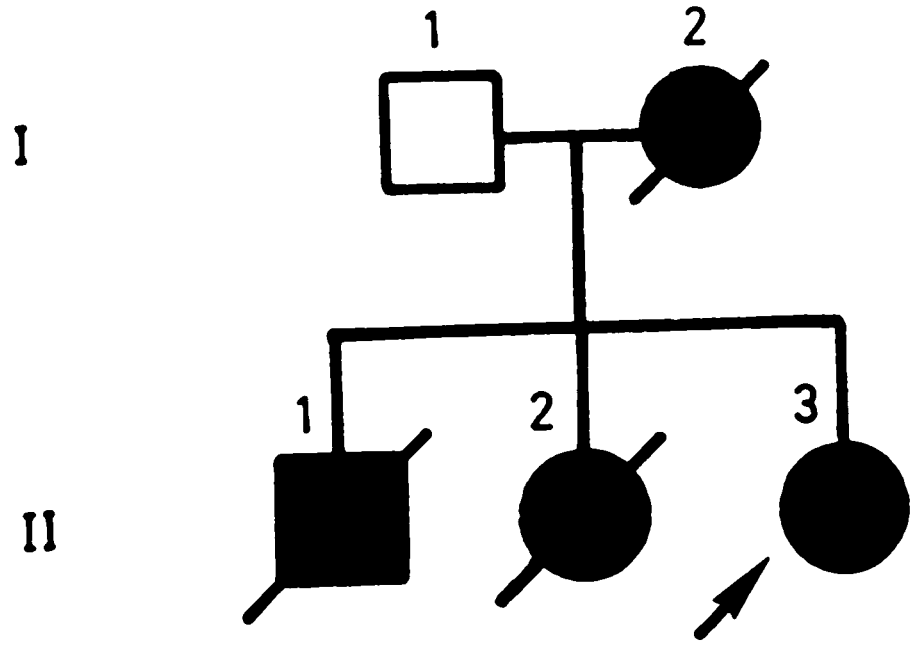


Figure 6.1

Pedigree of family 6. Patients I.2, II.1, and III.3 all had similar disease to the proband, apart from age of onset. They died aged 35, 15, and 10 from respiratory failure. Patient II.3 had progressive pain on exercise and weakness from the age of 17 and is now wheelchair bound.

6.3 Methods

Gross deletions and insertions in the predominant population of mtDNA in blood were excluded by doing a Bam HI digest, and probing with purifiedtotal mtDNA. Five restriction enzyme digests (Alu I, Dde I, Hae III, Hinf I and Taq I) were performed and probed with 11 regional probes corresponding to 83% of the protein reading frames (probes 3 to 13 of figure 5.1). Regional probes were not available for the remaining 17% of the reading frames. Further restriction digests were performed in the event of a site change in order to determine the likely point mutation.

6.4 Patients

Ten patients with mitochondrial myopathy were studied. Details of their clinical features, together with the results of histological and biochemical investigations are shown in table 6.1. Nine of the patients' mothers were alive and available for study. One had died from mitochondrial myopathy, and in one case the mother was affected, as demonstrated by histological criteria, and probably both sibs (fig 6.1). Six patients have been reported previously, as indicated. Normal subjects were included as controls.

6.5 Results and discussion

The restriction fragment sizes predicted from the human mitochondrial DNA sequence were detected for each combination of restriction enzyme and probe. The smallest fragment size detected was 116 bases. In seven patients the restriction fragments detected did not differ from those predicted from the known sequence and previous population polymorphism data. These results exclude the existence of major rearrangement insertions or deletions of greater than 60 base pairs for the predominant population of mitochondrial genomes in blood within the region of mtDNA examined. They also exclude point mutations within the 5% of this portion of mtDNA, which comprised the restriction sites surveyed.

New mutations were identified in three patients and in two of their mothers. In patient 1 and his mother, digestion with Alu I resulted in two new fragments of 375 and 966 bases replacing the normal 1.34 kb fragment. These were shown by probes 8 and 9, respectively (fig 6.2). Because transitions occur much more frequently than transversions, the most likely position can be inferred by searching the semi sites. This suggests a new

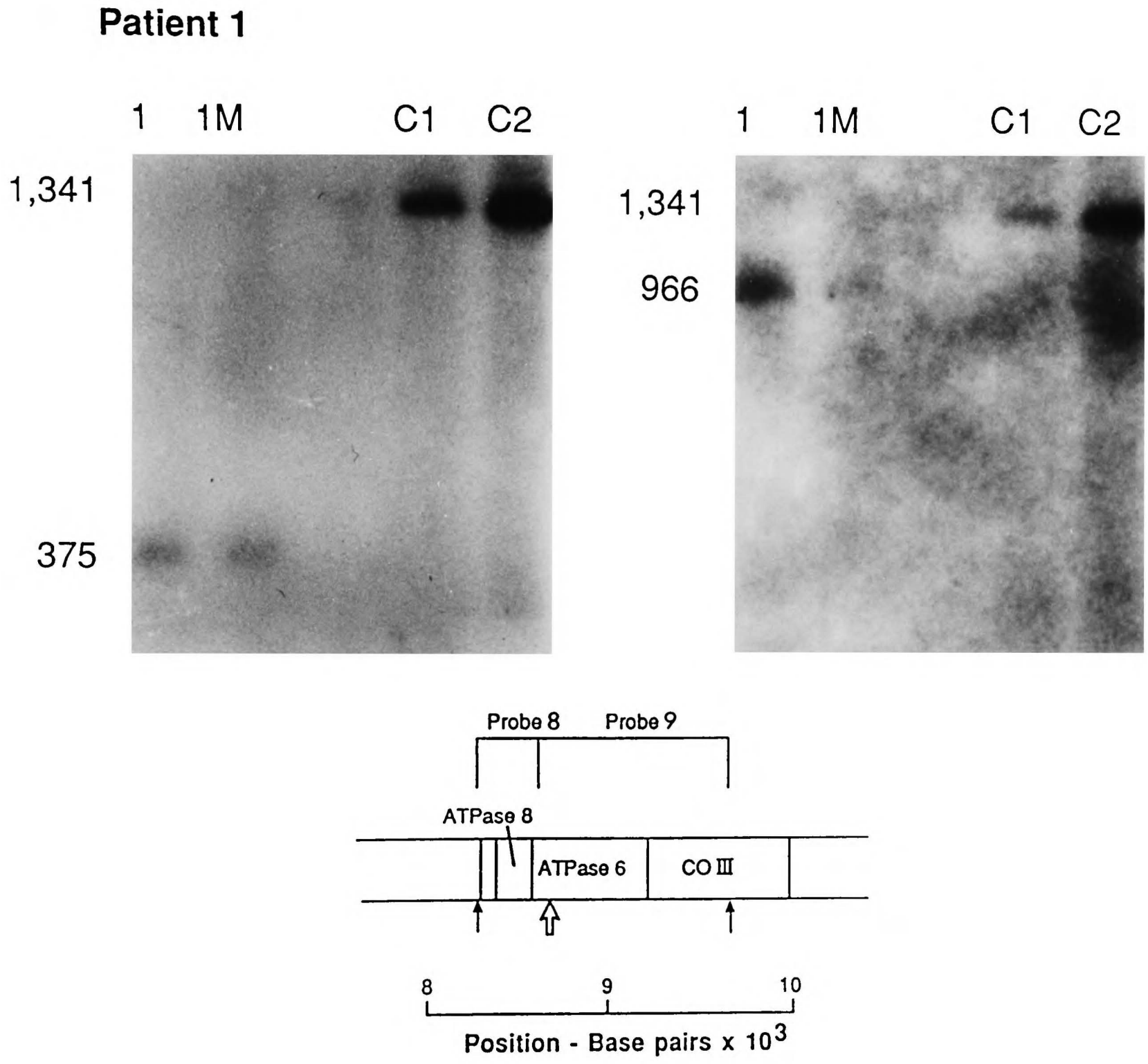


Figure 6.2

Site changes in case 1. Autoradiographs of AluI digest of case 1 (1), his mother (1M), and two controls (C1, C2), hybridised with (left) probe 8 showing replacement of the normal 1,341 fragment with a 375 fragment and (right) probe 9 showing replacement of the 1,341 fragment with a 966 fragment. (below) Restriction map of bases 8,305 to 9,645 of the mitochondrial genome.
Key: ↑ : expected Alu 1 site ⇧ : new Alu 1 site

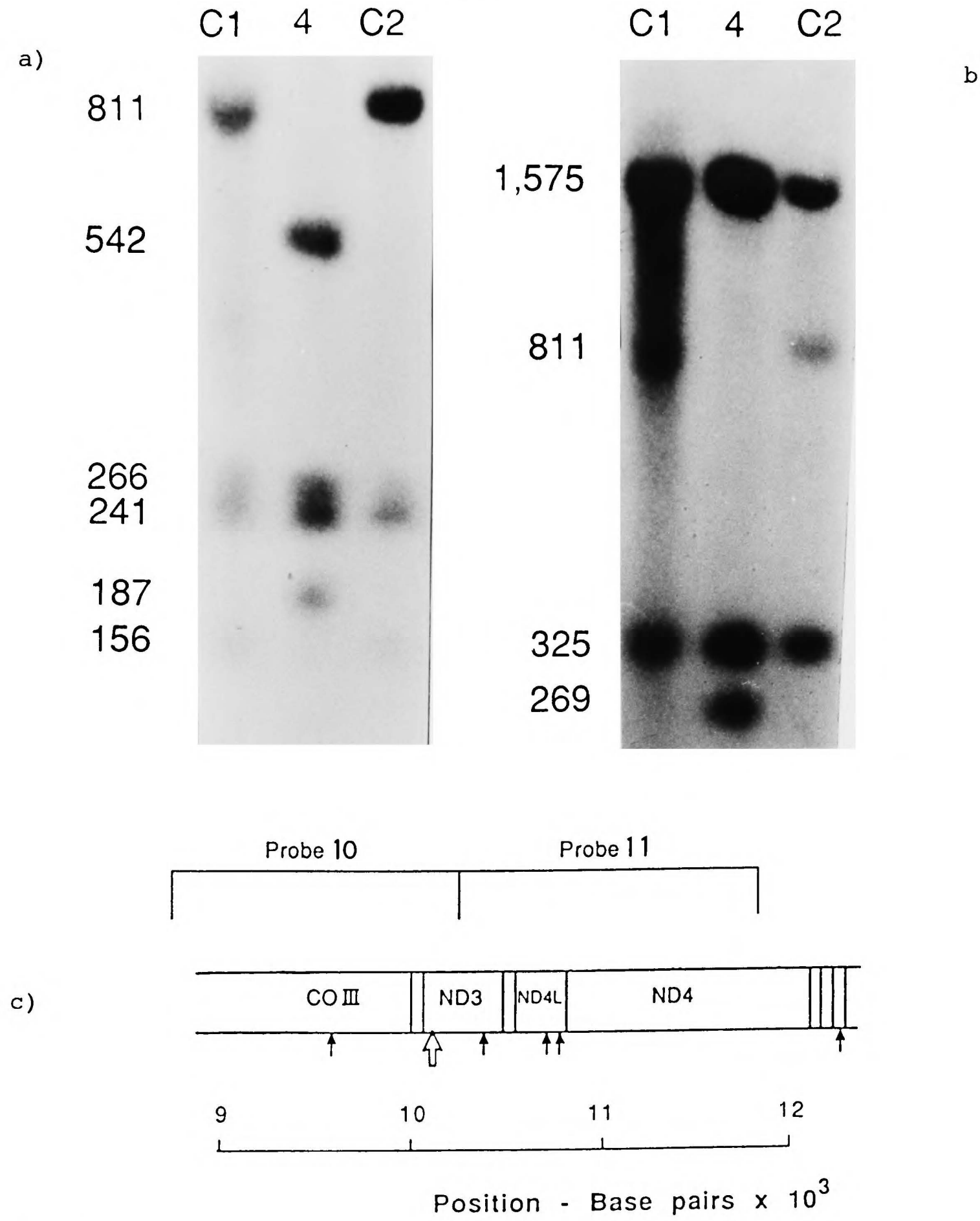


Figure 6.3

Site changes in case 4. Autoradiographs of a) HaeIII digest of case 4 (4) with two controls (C1, C2) hybridised with probe 10 to show replacement of normal 811 fragment by 542 and of normal 187 fragment by 156. b) Digest with HaeIII hybridised with probe 11 to show replacement of 811 fragment by 269 fragment. c) restriction map of bases 7975 to 13 705 of the human mitochondrial genome.

↑ : Expected Hae III site ⇧ : New Hae III site

Alu I site at 8679 base pairs, in the region coding for ATPase subunit 6, caused by an A-G transition which would not result in an amino acid change.

Digestion of DNA from patient 4, his sister, and his mother with Hae III showed one new polymorphism and one which has been described previously. The normal 811 base fragment was divided into 542 and 269 base pairs at a new Hae III site shown by probes 10 and 11, respectively (fig 6.3a,b). A double digest with Hae III and Hinf I suggested that the mutation creating the new Hae III site was located at 10,097 base pairs. Secondly, the normal 156 base Hae III fragment was replaced by a 187 base fragment (fig 6.3a), as has previously been described. This was consistent with a Hae III site loss at position 8995. In addition, digestion with Bst NI and hybridisation with probe 12 showed replacement of the normal 4,713 and 1,018 base fragments by two fragments of size 3,608 and 2,123 bases. This, again, suggests a Bst NI site gain around 10,097, and a Bst NI site loss around 8,995. This indicates that the most likely point mutation at 10,097 is an A-G transition lying within the recognition sites of both Hae III and Bst NI. This is the third base in a codon in the reading frame of ND3 and would not result in an amino acid change. Similarly, the most likely cause of the Bst NI and Hae III site losses around 8994 is a G-A transition either at position 8,994 or at 8,995. The former is the third base in a codon in the reading frame of ATPase 6, and would not result in an amino acid change; the latter would result in replacement of alanine with threonine.

In patient 3, digestion with Dde I and hybridisation with probe 13 showed a replacement of the 292 band by a 238 band. This site again could be located at either 15 738 or 15 945, and the latter was excluded by obtaining normal restriction fragments with Sty I. Thus, the most likely mutation is a G-A transition at 15 738, which would give rise to replacement of an isoleucine residue with valine.

Neither of these two amino acid changes is likely to affect the properties of the proteins substantially. However, their true location and significance must await further analysis by DNA sequencing, immunoelectrophoresis of protein products and funtional studies.

Two of the three new mutations found in this study were present in asymptomatic mothers of two patients and in one sib, and could therefore be uncommon population polymorphisms of no pathological significance. However, clinical studies suggest that penetrance is variable and apparently unaffected relatives may have histologically abnormal mitochondria. In addition, age of

onset of symptoms may be very variable even within a family (case 10). Neither of these parents has been investigated, and even muscle biopsy could not unequivocally exclude a mild defect in an unbiopsied muscle. There have been only two previously reported DNA studies of mitochondrial myopathy (Wallace et al. 1989, Holt et al. 1988a). Although they identified population polymorphisms, they did not find any alterations which they considered to be of pathological significance. Both of these studies used total mitochondrial DNA as a probe, which may give ambiguous results. Our study has taken this a step further by using a series of probes to give more regional detail over a range of patients and has excluded deletions, insertions, or rearrangements of 60 base pairs or above from 83% of the protein reading frames in the predominant population on mtDNA in blood (figure 5.1). Regional probes were not available for the remaining 17%, as this region is peculiarly resistant to cloning. This region was investigated using purified total mitochondrial DNA as probe, but in considerably less detail.

There are a number of possible explanations for this negative result. In three cases (patients 3,4 and 5) with Kearn's Sayre syndrome, Southern hybridisation of muscle DNA which subsequently became available showed large deletions (see Chapter 8). In one case, re-probing the BamH I digest with total mtDNA and over-exposing the autoradiograph showed that deleted genomes were present at low levels in blood, but had passed undetected in the present study. In four more cases, Southern hybridisation of muscle DNA which subsequently became available did not reveal any major rearrangements. In the three cases in whom muscle data is still unavailable, rearrangements of muscle mtDNA remains a possibility as mtDNA extracted from white cells is not necessarily identical to that found in skeletal muscle. For the rest, point mutations of mtDNA may be causative, particularly in the case of patient 6. On the other hand, nuclear mutations may be causative in some cases. The 13 reading frames of the mitochondrial genome encode only a small minority of the known protein subunits of the electron transport chain (table 3.1). The remainder are encoded by nuclear genes, and in addition there must be many regulatory genes involved in assembly. These figures suggest that most cases of mitochondrial myopathy are the result of mutations in nuclear genes and would occur sporadically or follow an autosomal recessive inheritance pattern. The majority of biochemical defects in man have such a mode of inheritance (McKusick 1986).

The patients in this study were a representative group of cases of mitochondrial myopathy occurring after the neonatal period and in no case was the diagnosis in doubt.

6.6 Conclusion

In conclusion, these results show that large deletions or insertions are not present in the major population of blood mtDNA of a representative selection of patients with mitochondrial myopathy.

Changes in restriction fragment size explicable by point mutations or small structural rearrangements were observed in three out of 10 patients. It is likely that these represent silent population polymorphisms.

Chapter 7: Results

Tandem duplications of mtDNA

7.1 Summary

Two patients with direct tandem duplications of mitochondrial DNA (mtDNA) and mitochondrial myopathy are described. The breakpoint regions between duplicated segments were amplified using the polymerase chain reaction (PCR), cloned and sequenced. The distribution of normal and abnormal genomes in different tissues was investigated using Southern hybridisation, and in different cells within the same tissue using PCR.

In each case the gene for cytochrome oxidase subunit I (MTCOX1) was interrupted, creating reading frames which if transcribed and translated would result in truncated versions of this peptide. The Picolli sequence (Picolli et al. 1984) was found within 13bp of the break in each case, suggesting a possible causal role in rearrangements of mtDNA.

Heteroplasmy and mosaicism for the abnormal mtDNA population was apparent. Preliminary data also suggest that high molecular weight re-arrangements of the duplicated region are present in all tissues.

7.2 Introduction

After completion of the restriction enzyme analysis described in chapter 6, venous samples on two further patients were obtained in whom there was clear evidence for a re-arrangement of mtDNA. This chapter describes the mapping data obtained by using restriction enzyme analysis of DNA from whole blood and of PCR products, and subsequent sequence analysis of the junction fragment between duplicated segments. The hypothesis that these duplicated genomes caused the phenotype was investigated by examining the distribution of duplicated genomes in various tissues using Southern hybridisation, and by RNA analysis. This included Northern blotting and cDNA sequencing.

In order to investigate the origins of the duplicated mtDNAs, their distribution in different cells within a tissue was documented using PCR.

7.3 Patients:

Patient 1 (fig 7.1) presented with diabetes mellitus and short stature at the age of seven. Classical features of KSS developed, with ptosis, external ophthalmoplegia, retinopathy, ataxia, proximal muscle weakness and

Fig 7.1 Patient 1

Fig 7.2 Patient 2

deafness. Muscle biopsy showed classical ragged red fibres. She later developed a cardiac conduction defect, and hypokalaemia, probably because of a renal tubular defect. She died suddenly probably as a result of this combination.

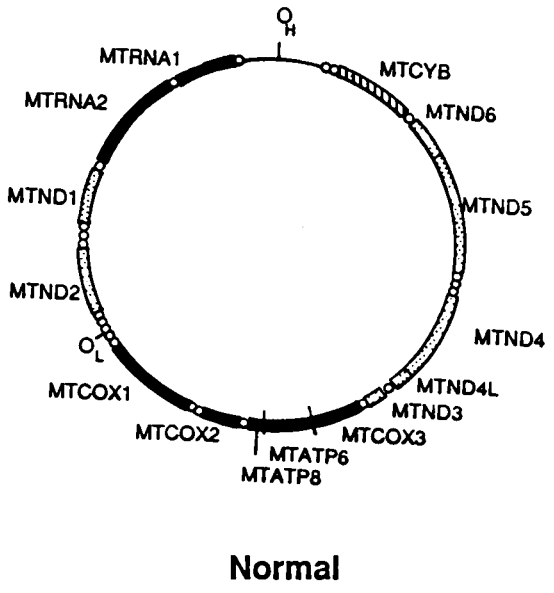
In patient 2 (fig 7.2) mitochondrial myopathy was diagnosed at the age of 12 when she had developed progressive ataxia, dysarthria,muscle weakness, external ophthalmoplegia, ptosis and mental retardation. Muscle biopsy early in her illness did not reveal any specific histological abnormality. She subsequently developed a transient hemiparesis following a stroke-like episode.

7.4 Restriction enzyme analysis

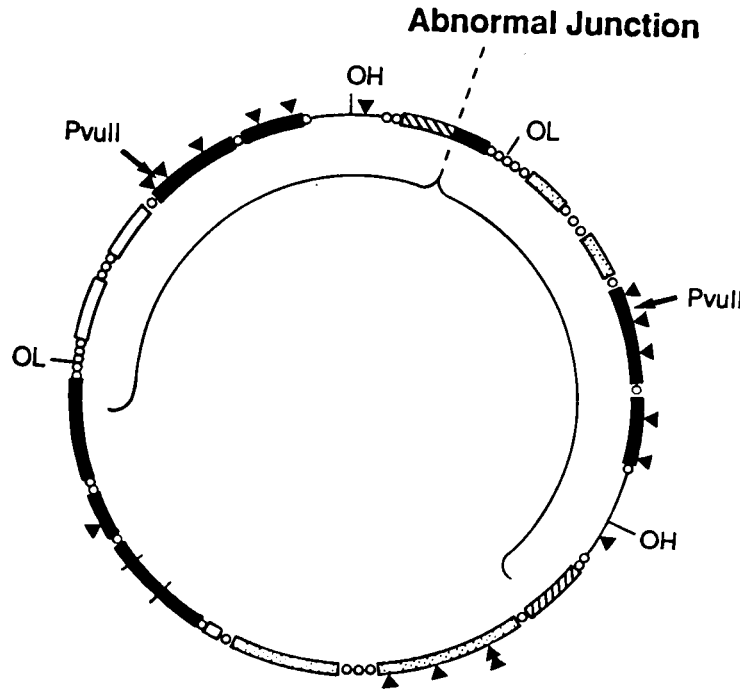
Restriction enzyme analysis was done on total cellular DNA extracted from whole blood from the eighteen patients, and from the mothers, unaffected siblings, and one of the fathers of patients 1 and 2, probing with either purified whole human placental mtDNA or one of fourteen small fragments of HeLa cell mtDNA in bacteriophage M13 (Fig 7.3a, 7.4). MtDNA contains a single restriction site from Bam HI which linearises the genome, and hybridisation with total mtDNA reveals a single 16.5 kb band. An extra band 8 kb larger than the normal band was present in patients 1 and 2, but not in unaffected family members, suggesting that either an insertion or a duplication was present (Fig 2). Similar bands were observed with undigested DNA. No abnormalities were seen in the remaining sixteen subjects.

In patient 1 digestion with Ava II revealed an additional abnormal band of 4.6 kb (Fig 7.5) which hybridised with probes 13, 14, 3, 4, and 5, suggesting that the additional DNA was mitochondrial in origin. Since these probes hybridised with portions of the genome usually distant from each other, this finding indicates an abnormal junction between these two region. Furthermore, since the 4.6 kb band is shorter than the 8 kb extra DNA, the latter must contain an internal Ava II site. An insertion was excluded by the absence of a second abnormal fragment. Hence a tandem duplication was present, and was confirmed by the results of digestion with Bst NI, Eco RI, Hpa I, and Sac I, and hybridisation with total mtDNA.

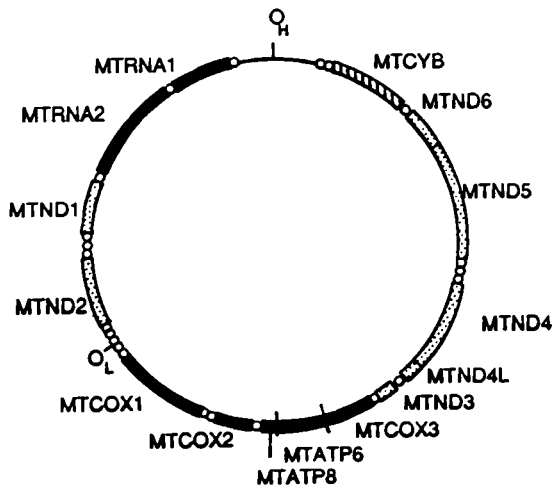
Normal mtDNA contains a single Pvu II site which is situated within the region thought to be duplicated in both patients. Digestion with this enzyme and hybridisation with total mtDNA revealed, in addition to the normal 16.5 kb



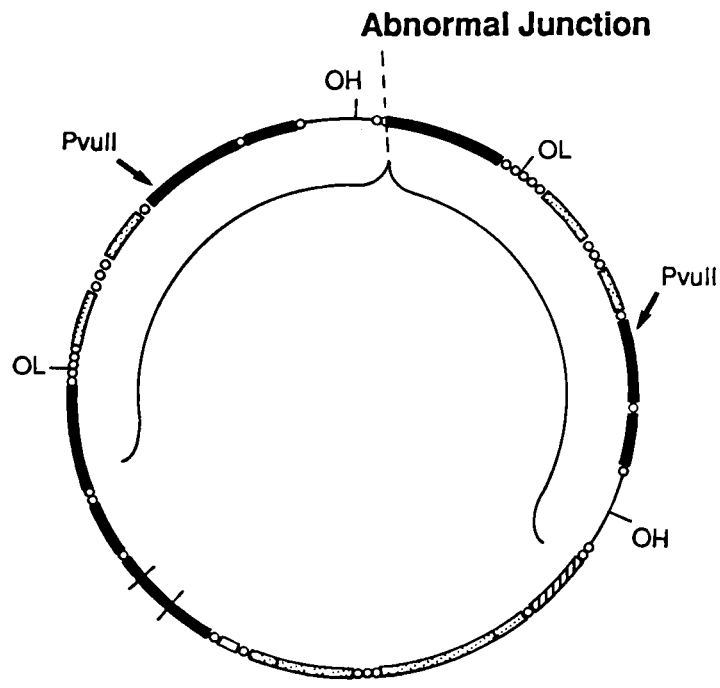
Normal



Patient 1



Normal



Patient 2

Fig 7.3
Diagram to show the location of mtDNA duplications in patients 1 and 2. In each case the bracketed regions represent the duplicated portion.
O_H: Origin of replication of the heavy strand. For genes involved in the duplicated region see text.

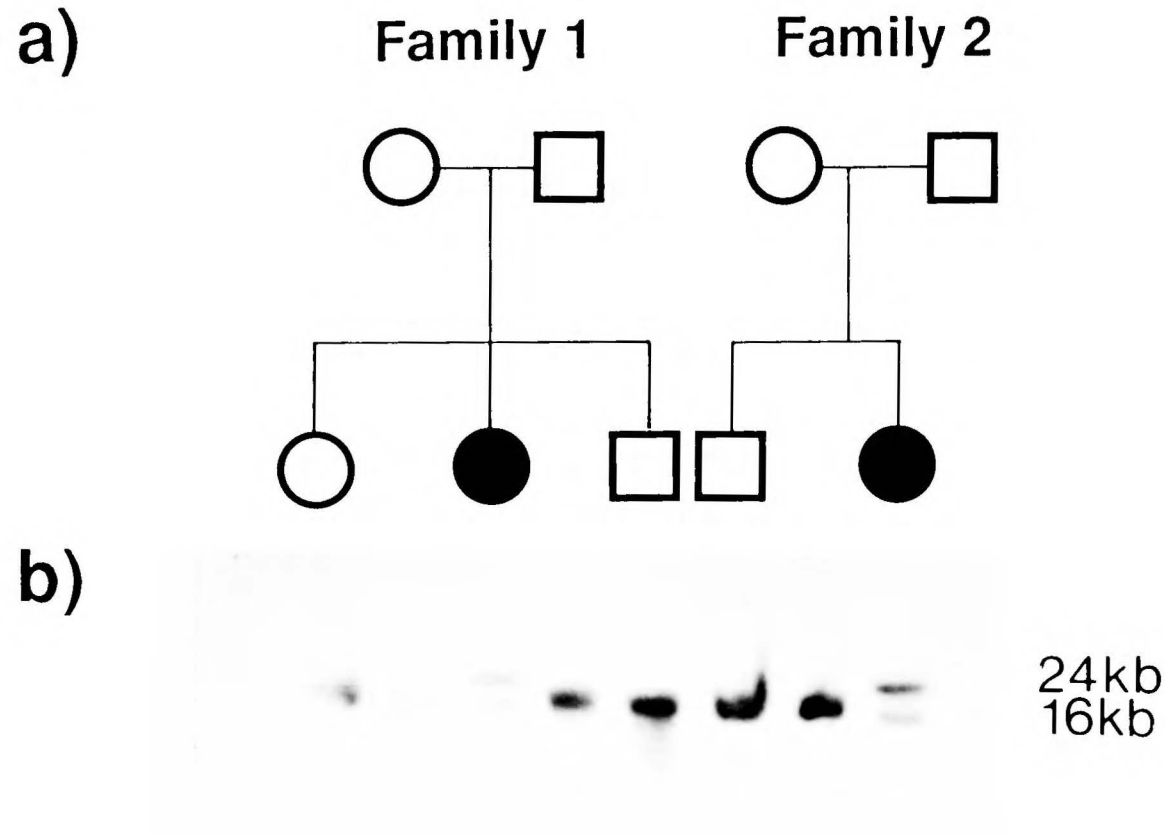


Fig 7.4. Restriction enzyme analysis and pedigree of 2 patients with mitochondrial myopathy and their families.

●, affected female; ○, unaffected female; □, unaffected male (No DNA available from father in family 2).

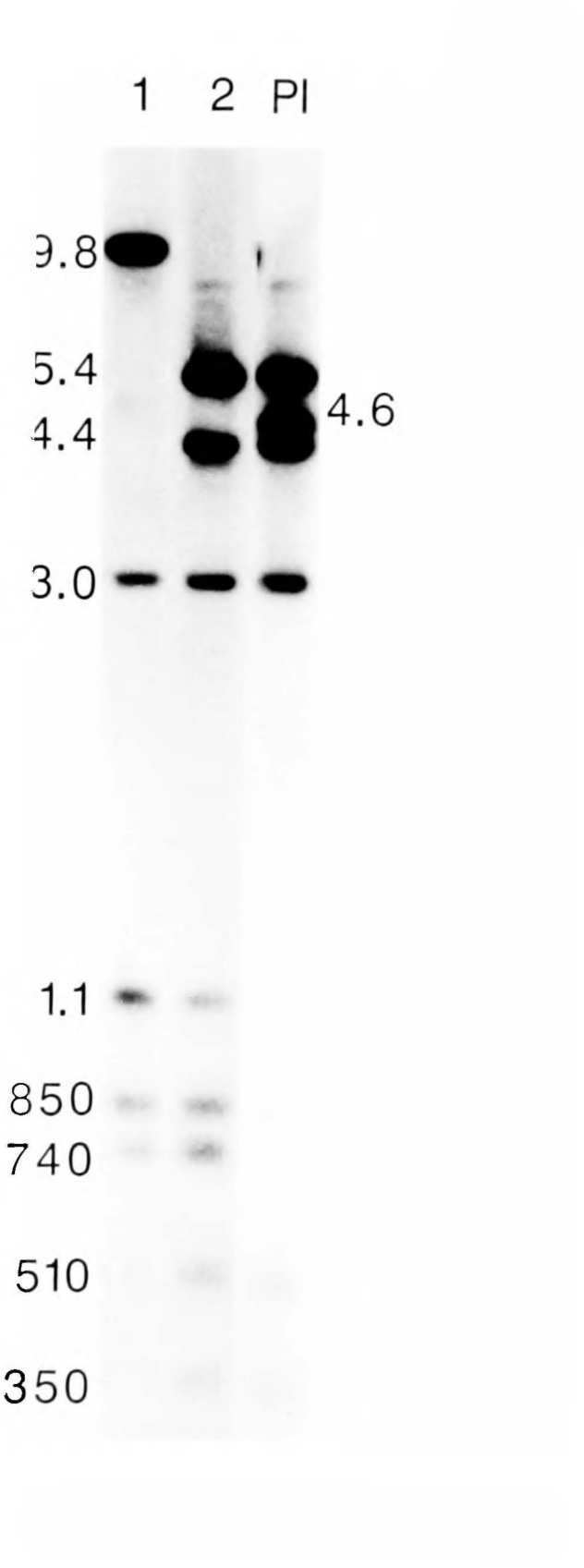


Fig 7.5. Restriction enzyme analysis with Ava II.

Hybridisation with purified mitochondrial DNA may reveal various polymorphisms, two of which are shown (morphs 1 and 2). Patient 1 was a morph 2 but with an extra 4-6 kb band. 1 = morph 1; 2 = morph 2; P1 = patient 1.

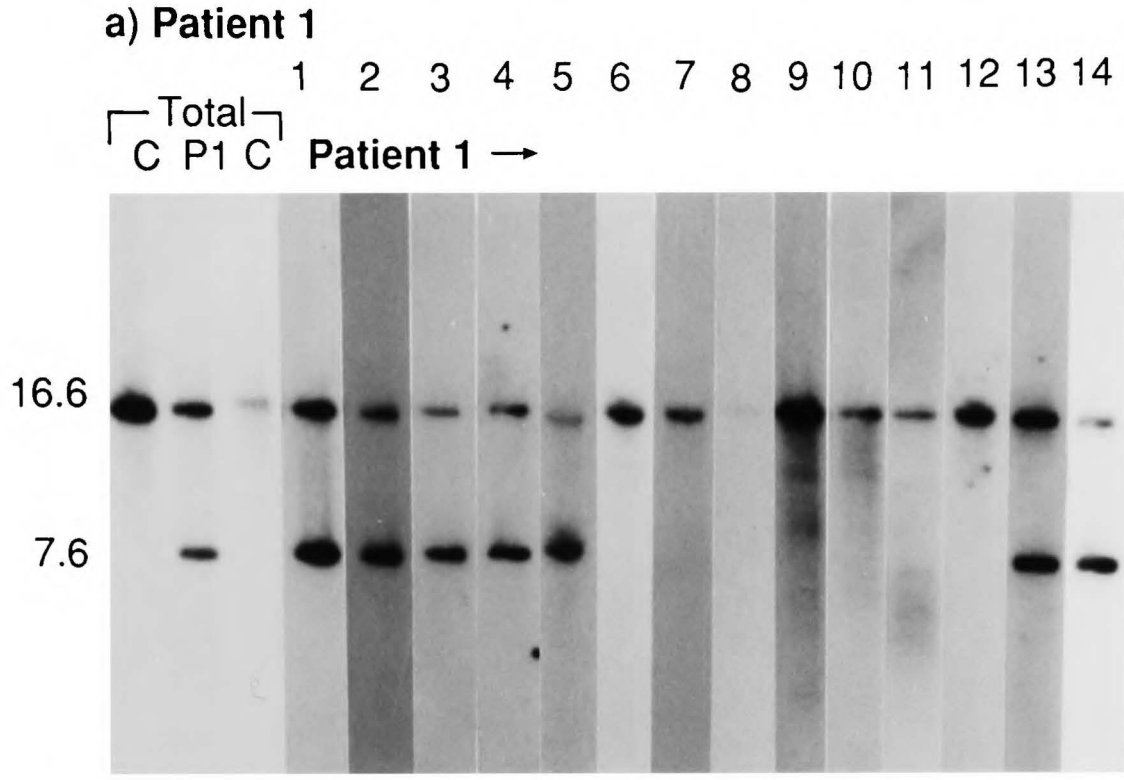
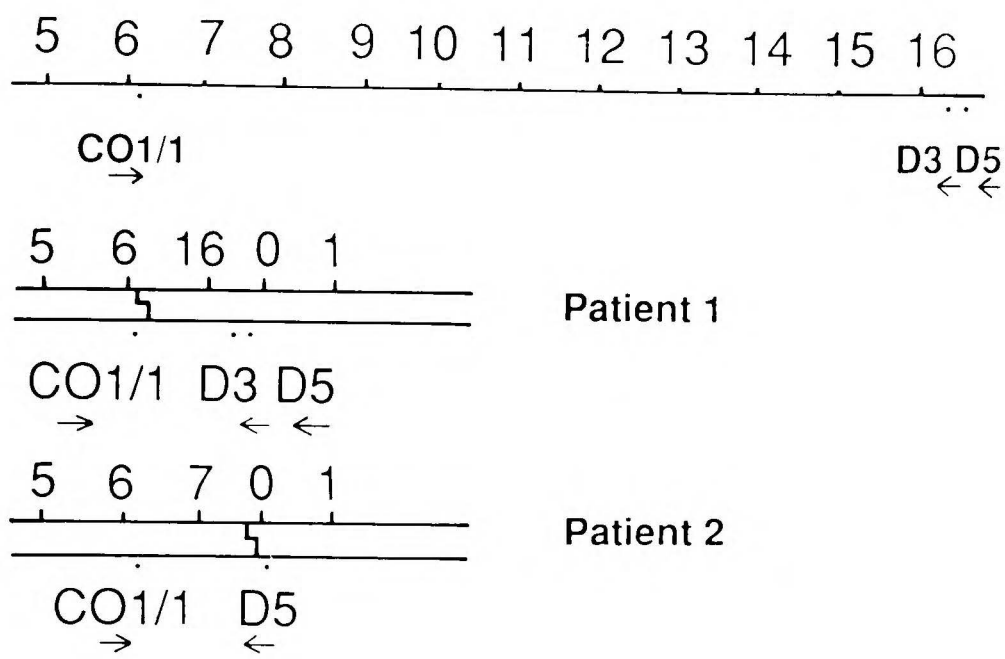


Fig 7.6. Position of tandem duplications in patient 1(a) and patient 2(b).

Patient 1, abnormal 7.6kb fragment hybridised with probes 13, 14 and 1-5; patient 2, abnormal 8.0 kb fragment hybridised with probes 14 and 1-6. C = control; P1 = patient 1; P2 = patient 2; 1-14 = hybridisation with mitochondrial probes 1-14 (see Fig 1); total = hybridisation with purified mitochondrial DNA. Bands are present but have not reproduced well at 16.6 kb in lane 8 (patient 1) and in lanes 2, 5, 13 and 14 (patient 2), and at 8.0 kb in lanes 1, 2 and 14 (patient 2).

a



b

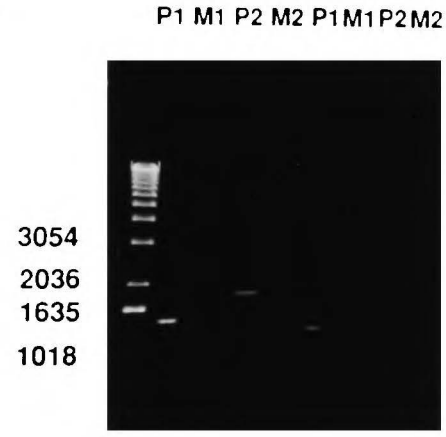


Fig 7.7. Amplified products of PCR with oligonucleotide primers either side of the abnormal junction: location of primers COI and D5 and expected products (a); and gel electrophoresis of products with size markers (b).

In (a) = position of primers, = direction of primers; upper bar = positions relative to reference sequence in normal controls; lower bars = approximate location of abnormal junctions. In (b) P1 = patient 1; M1 = patient 1's mother; P2 = patient 2; M2 = patient 2's mother.

band, an abnormal band of about 8 kb. This band represents the full length of the extra DNA, and suggests that the duplicated DNA contained a Pvu II site (Figs 7.3b, 7.6). This digest was hybridised with each of probes 1-14 (figs 5.1 and 7.6) to localise the duplication. The results of the hybridisation suggested that the duplicated portion was situated at a position equivalent to between 15,000 and 6,000 bases of the reference sequence (see Fig 7.3) in patient 1 (Fig 7.6a) and at between about 16,000 and 7,000 bases in patient 2 (Fig 7.6b).

The PCR was used in each case to amplify across the junctions between repeats to localise the junction more precisely. Amplification with primers COI/1 (bases 6004 to 6023 of the reference sequence) and D5 (complementary to bases 16,255 to 16,274) produced a 1.5 kb fragment in patient 1 and 1.8 kb fragment in patient 2 (Fig 7.7). PCR with this pair of primers in normal controls did not give rise to any products since the primers were too distant from each other for the amplification reaction to occur under the conditions used. This finding suggests that the duplications are 7.6 (patient 1) and 8.0 (patient 2) kb pairs in length. Restriction mapping of the amplified products placed the duplicated regions at between positions 6033 (Bst NI site present) and 6203 (Hind III site absent) and about 14 960-15 130 for patient 1, and 7204 (Hpa II site present) and 7376 (Bst NI site absent) and about 15 800 and 16 129 (Kpn I site absent) for patient 2. The precise location of the abnormal junctions can only be fully resolved by sequencing.

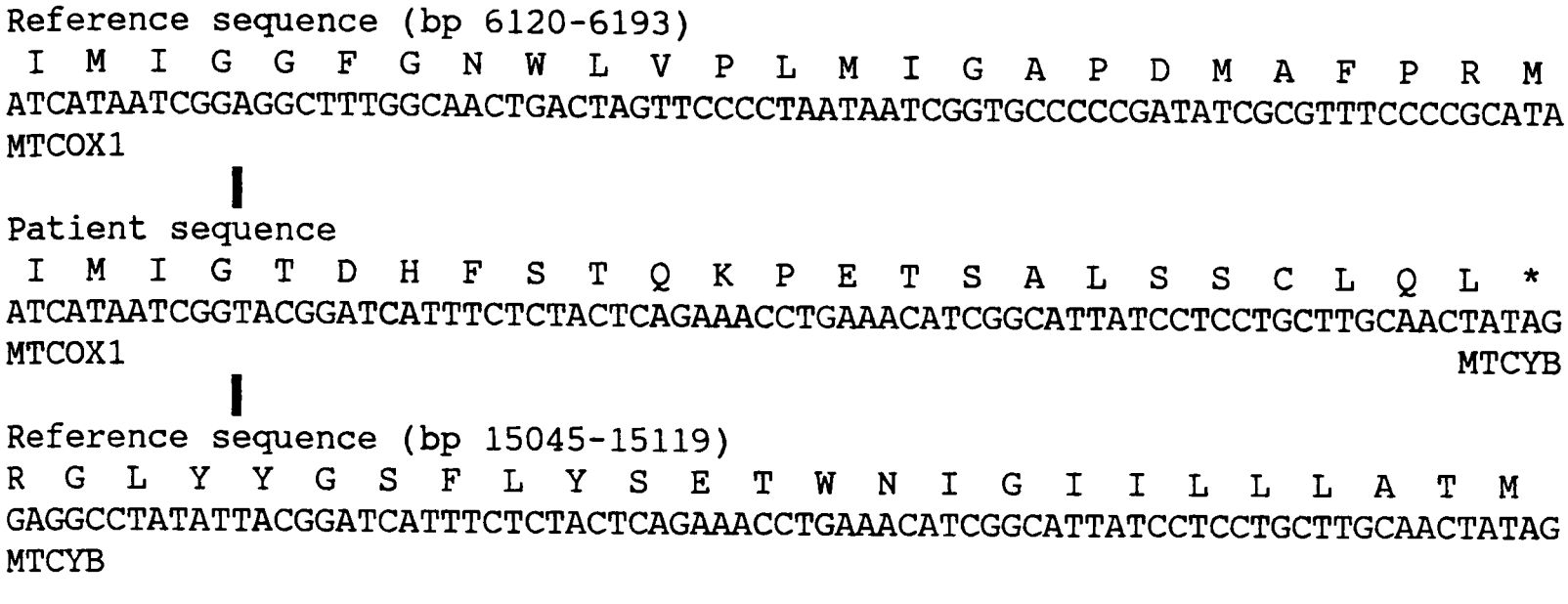
Amplification of DNA from whole blood of family members did not show duplicated mtDNA in any of them.

7.5 Sequence Analysis

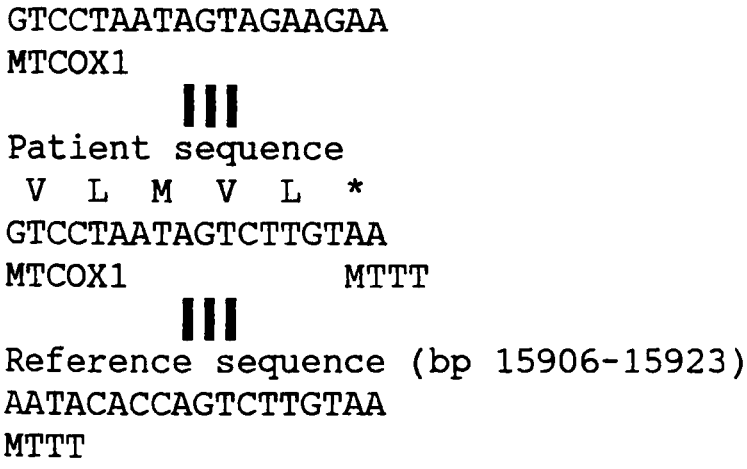
A single clone was obtained for each patient. Sequencing confirmed the preliminary mapping data for both patients (fig 7.8). In both cases the gene for cytochrome oxidase subunit I is interrupted: by apocytochrome b in patient 1, and by $tRNA^{thr}$ in patient 2. The mtDNA of both patients is therefore duplicated for about half of the total length of DNA, and carries two complete copies of the genes for NADH dehydrogenase subunits 1 and 2, for both rRNAs, for several tRNAs, the D-loop and both origins of replication. In the case of patient 1, the reading frame for cytochrome oxidase subunit 1 (MTCOX1) was interrupted at base no 6,130 with base no 15,056 of apocytochrome b (MTCYB) sequence out

Figure 7.8

a) Patient 1



b) Patient 2



Legend to Fig 7.8

Sequence data: Reference sequence of parent strands above and below the sequence obtained and inferred amino acid sequence of any translated peptide in a) patient 1: Cytochrome oxidase subunit1 sequence at bp 6130 adjoins bp 15056 (apocytochrome B) and b) Patient 2: at bp 7354 Cytochrome oxidase subunit1 sequence adjoins bp 15913

Key: ▌ represents breakpoint (patient 1) or homologous region (patient 2), MTCOX1: Cytochrome oxidase subunit1, MTCYB: apocytochrome B, MTTT: $tRNA^{thr}$.

* stop codon

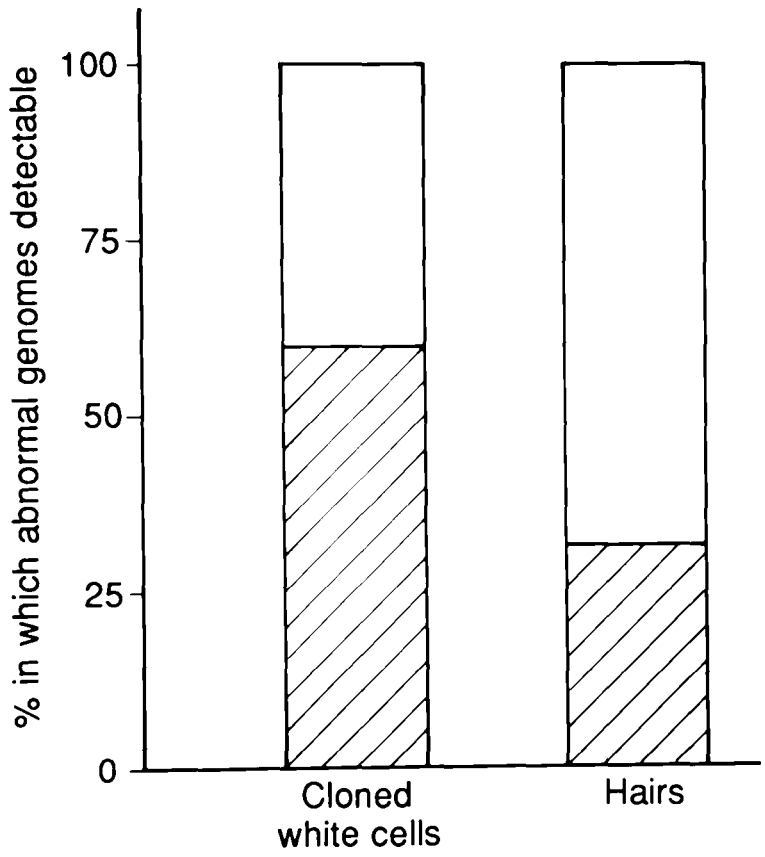
of frame without any intervening sequence (fig 7.8). If transcribed and translated this would result in a truncated peptide consisting of the first 76 residues of MTCOX1 sequence followed by 20 abnormal residues unrelated to the normal MTCOX1 or MTCYB amino acid sequence before the first stop codon is generated. In

patient 2, MTCOX1 sequence is interrupted after base no 7,354 with base no 15,913 of the $tRNA^{thr}$ sequence without any intervening sequence. There was a homologous portion 3 base pairs in length in both parent strands. If transcribed and translated this would result in a peptide corresponding to the first 497 amino acid residues of MTCOX1 followed by one abnormal residue before the first stop codon is generated. In order to investigate the possibility that reciprocal deleted genomes might be present corresponding to the DNA portion effectively deleted between the duplicated segments PCR was also carried out using primers CO1/4 and Cytb2. If these genomes existed they should give rise to products 1.4 and 1.1 kbp in length. No product was obtained although each primer was able to generate product when used in other appropriate combinations.

7.6 Tissue distribution of duplicated genomes in patient 1.

Samples and Southern hybridisation: Total cellular DNA was extracted from whole blood and from EBV transformed lymphocyte lines, and from muscle, urinary epithelium and fibroblasts in patient 1. Subsequently autopsy samples also became available. DNA was digested with BamHI (Amersham), electrophoresed in 0.8% agarose gels, Southern blotted and probed with purified total mtDNA

Densitometry revealed that normal and abnormal mtDNAs comprised approximately 32 and 43% of the total mtDNAs in blood in both cases 1 and 2 respectively (table 7.1). Patient 1 had 20-25% abnormal genomes in muscle. No muscle tissue was available for patient 2. Southern hybridisation failed to detect abnormal genomes in hair, fibroblasts, or bladder epithelial cells, but these were detectable using PCR (see table 7.1), suggesting that they were present at low levels. Post mortem samples have recently become available from patient 1. The duplicated genomes were present in all tissues examined: skeletal muscle, heart brain, liver, pancreas and ovary. In addition, high molecular weight species which hybridised to purified total mitochondrial DNA were also present in undigested DNA from all tissues. These were also found to be present in blood, only at very low levels, apparent only by over-exposing the autoradiograph. One of these (size approx 32-40 kb) was also present in lymphocyte lines from both patients and controls, and was probably a dimer of



Legend to Fig 7.9

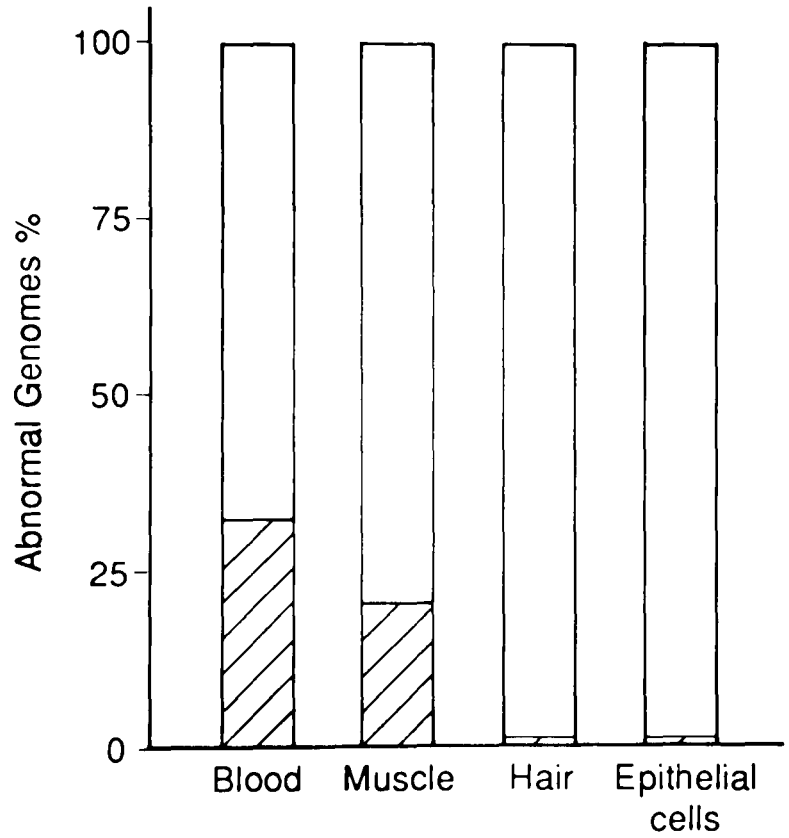
Bar charts showing varying proportions of duplicated mtDNAs in Patient 1 in different clones within a tissue (vertical axis: percentage of single hairs or white cell clones in which duplication sequence was detectable)

Hatched regions:duplicated genomes

Unhatched regions: normal genomes

Table 7.1: Proportion of duplicated mtDNAs in various tissues.

Tissue		Mitochondrial genome		Proportion of clones in which duplication detectable
		% duplicated	%normal	
Patient 1	Blood	32%	68%	
	Cloned granulocytes	NA	NA	3/5 (60%)
	Muscle	20%	80%	
	Hair - Pooled	<5%	>90%	
	- Individual	NA	NA	5/16 (31%)
	Urinary epithelial cells	<5%	>95%	
Patient 2	Blood	43%	57%	



Legend to Fig 7.10
Bar charts showing varying proportions of duplicated mtDNAs in Patient 1 in different tissues by laser densitometry (vertical axis: percentage of duplicated mtDNAs)
Hatched regions:duplicated genomes
Unhatched regions: normal genomes

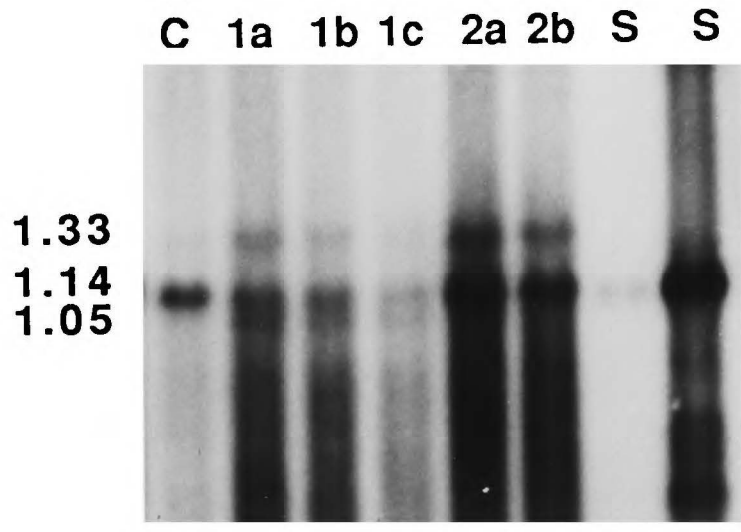
the normal 16.5kb genome (it disappeared on digestion with any enzyme which normally cuts mtDNA such as Bam Hl or Pvu ll, and when excised from a gel and electro-eluted it could be digested to a 16.5kb band with Bam Hl). A second band ran just ahead of this in the two patients but not in controls, and was of slightly different size in the two patients. It hybridized to probes corresponding to the duplicated segment, and with the junction-specific oligonucleotide SAO (patient 1 only), but not with probes corresponding to the non-duplicated segment. It could be digested by Pvu ll but not by Bam Hl. The simplest explanation is that this is that it represents a re-arrangement of the duplicated mtDNA, for instance, four copies of the duplicated segment joined end to end. This is consistent with the difference in size of the bands between patients. Further work is required to clarify this, and to accurately quantitate it and the 24kb band relative to normal mtDNA in different tissues.

7.7 RNA analysis

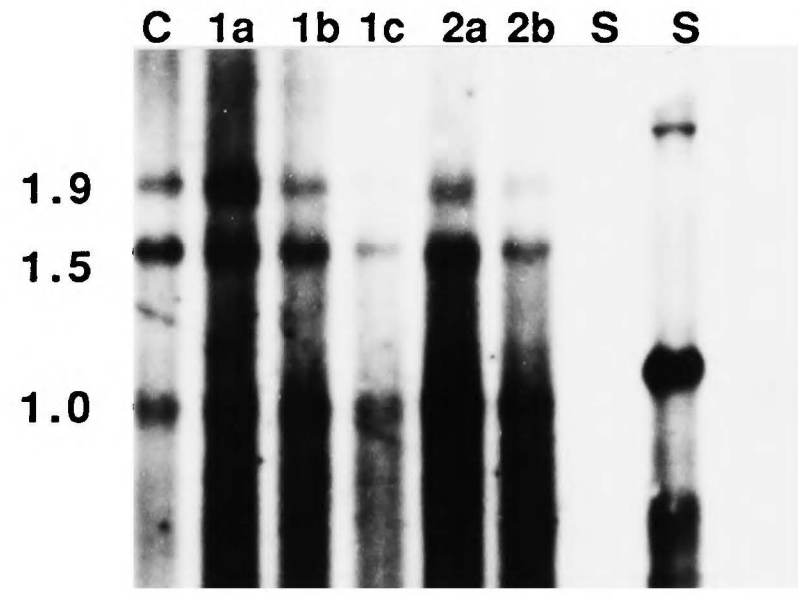
Northern analysis of total cellular RNA from lymphocyte lines in both patients, and from skeletal muscle, heart, brain and liver in patient 1. This was hybridised to regional probes 5, 13 and 1 (corresponding to the MTCOX1, MTCYB and D-loop regions respectively, see fig 7.3) and to an end labelled oligonucleotide designed to be complementary to the expected transcript from this patient's junction region (SAO). Fig 7.10 shows that probe 13 hybridises to the normal cytochrome b transcript (RNA 11 Attardi, 1984) which is 1.14 kb in length. There is an additional abnormal transcript in patient 1 of approximately 1.06 kb, whose size corresponds to the predicted RNA from the region of the abnormal junction. This hybridised with the end labelled junction oligonucleotide, SAO. Probe 5 revealed only the normal transcripts (1.86, 1.54 and 1.04 or RNAs 6, 9 and 12 respectively). There was an additional transcript of about 1.35 kb in all lanes, but particularly prominent in RNA from lymphocyte lines, which probably represents an unprocessed intermediate. No abnormal transcripts were detected on Northern analysis of lymphocyte RNA for patient 2.

cDNA was synthesized from RNA which had been treated with DNAse (section 5.1.8 and 5.9.3), and the product amplified with primers either side of the abnormal junction (tRNAY or CO1/1 and Cytb3 for patient 1, and tRNAY or CO1/3 and tRNAT for patient 2). In all cases a product of appropriate size was obtained using cDNA or total DNA, but not using the RNA preparation which had not been reverse transcribed (fig 7.13). This confirms that most or all of the

Fig 7.11: RNA analysis
a) Northern blot probed with regional mtDNA probe 13:
C: control
1a-c: different loadings of RNA extracted from lymphocyte lines from patient 1.
2a-b: different loadings of RNA extracted from lymphocyte lines from patient 2.
S: size markers
The expected band corresponding to RNA 11 is seen in all lanes. A second fainter band is seen in all lanes, probably corrsponding to an unprocessed precursor of this transcript. A band of approximately 1.05 is apparent in patient 1 but in no other lane.



b) Same filter as (a) probed with regional mtDNA probe 5:
Bands are seen at 1.0, 1.5 and 1.9 kb in all lanes, corresponding to predicted sizes for normal transcripts, RNA12, 9 and 6.



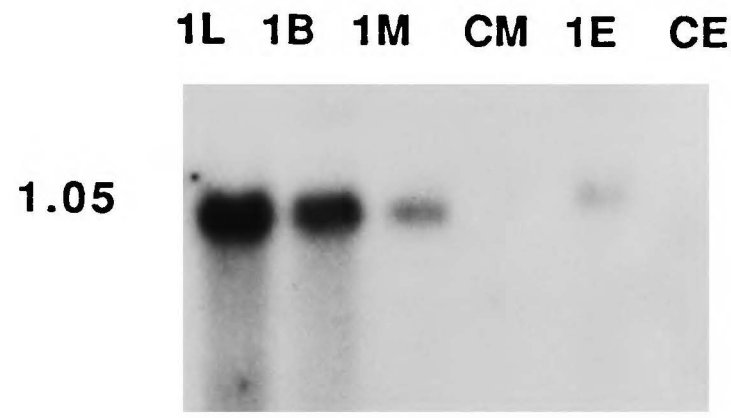


Fig 7.12: RNA analysis

Northern blot of RNA extracted from various tissues from patient 1 and control.
1L: Liver from patient 1
1B: Brain from patient 1
1C: Skeletal muscle from patient 1 from patient 1
CM: control muscle
1E: Lymphocyte lines from patient 1
CE: Control lymphocyte line.

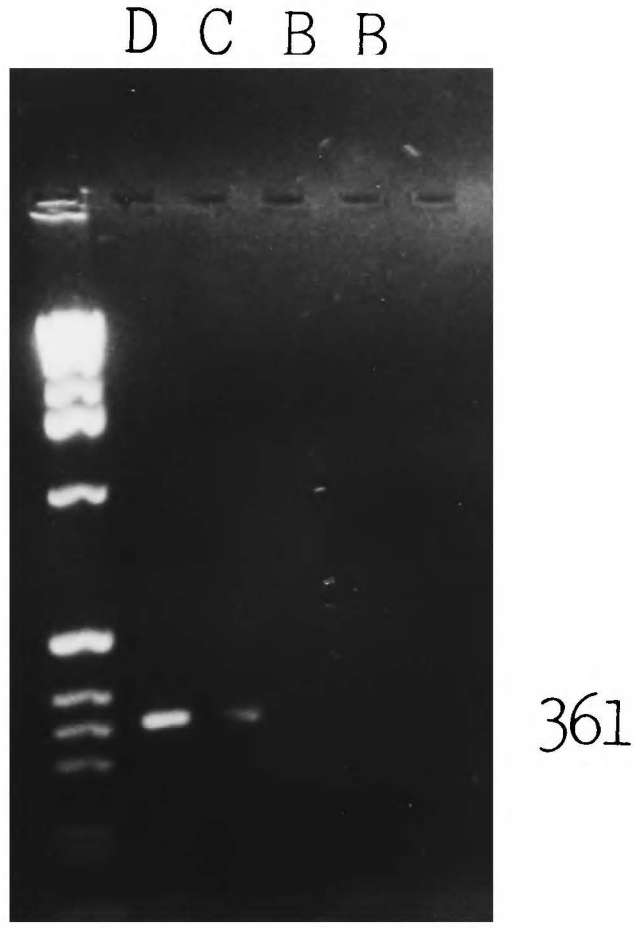


Fig 7.13: Analysis of cDNA

PCR product electrophoresed in a 1.5% agarose gel and stained with ethidium bromide, following amplification of cDNA synthesized from patient one's lymphocyte RNA with primers tRNAY and Cytb3.
First lane: size markers. The PCR template used in each lane was as follows:
D: Blood DNA from patient 1
C: cDNA as described above
B: DNAse treated RNA, exactly as C only without reverse transcriptase
B: Water blank

product arises from cDNA rather than from contaminating genomic DNA. The products tRNAY-Cytb3 for patient 1 and CO1/3-tRNAT for patient were cloned (one clone patient 1, two clones patient 2). Sequence analysis confirmed that the cDNA sequence was identical to that for the genomic fusion genes at the junction between duplicated segments, suggesting that it is indeed transcribed in both patients. The greater sensitivity of this method is demonstrated by its ability to detect the transcript from patient 2 in the absence of any signal on the Northern blot.

7.8 Proportion of duplicated mtDNAs in different cells within the same tissue

Clonality within a tissue was examined using PCR to detect duplicated genomes on granulocyte clones (Potter and Capellini 1983) and hairs (X inactivation data suggests that individual hairs arise from single cells, Gartler and Riggs 1983) in patient 1. Thirty cycles of PCR was carried out using primers across the abnormal junction (D5 or D3 and CO1/1 see fig 7.7), and with control primers CO1/1 and CO1/4 (fig 3.2) in a separate reaction to confirm the presence of normal MtDNA available for amplification. Where no duplication-specific signal was obtained after 30 cycles, 1/10 of the product was subjected to 30 more cycles of PCR. In no case did a signal appear after the second round. The relative quantities of PCR product from individual hairs and granulocyte clones suggested that the proportions of normal and abnormal mtDNAs varied in different cells within the same tissue.

These results suggest that this patient is a mosaic, ie different tissues contain different ratios of normal to abnormal genomes.

7.9 Discussion

We have studied two sporadic cases of Kearns-Sayre syndrome in whom Southern hybridisation showed heteroplasmy in both blood and muscle, with an abnormal population of mtDNAs which were 24kb in length. Restriction enzyme analysis using 14 regional human mtDNA probes in M13 showed that these were direct tandem duplications (Fig 7.8).

In both cases the duplicated regions included the D-loop (which contains both promoters and the origin of replication of the heavy strand), the reading frames for subunits 1 and 2 of NADH dehydrogenase (MTND 1 and 2), the genes for several transfer RNAs both ribosomal RNAs. Mapping data was confirmed by sequence analysis using the polymerase chain reaction (PCR) to amplify the

junction between the duplicated segments. In each case the gene for cytochrome oxidase subunit 1 (MTCOX1) was interrupted, with the gene for apocytochrome b out of frame in patient 1, and with $tRNA^{thr}$ gene in patient2. In the case of patient 1, the reading frame for MTCOX1 was interrupted after base no 6130 with base no 15056 of apocytochrome b (MTCYB) sequence out of frame without any intervening sequence (Fig 7.8).

As these abnormalities are specific to the two patients, and have not been observed in unaffected family members or in normals despite extensive population surveys (Brown et al. 1979, Johnson et al. 1983, Horai et al. 1984, Brega et al. 1986, Santachiara-Benerecetti et al. 1988), we believe that they are causative. We have shown that the fusion genes are transcribed, and if translated these would result in abnormal truncated subunits of CO1. It is possible that either abnormal transcripts or peptides could impair mitochondrial function by competing with the normal gene products. There is a precedent for this: one variety of cytoplasmic male sterility in Petunia is probably caused by the translation product of a duplication which results in a fusion gene (Young and Hanson 1987). Alternatively an excess of normal products from the duplicated regions could be harmful. The fact that the phenotype of plants (for example Small et al. 1989) and lizards (Moritz and Brown 1987) does not appear to be affected by some duplications makes the former slightly more likely. Further analysis of RNA and proteins may clarify this further.

Of the tissues investigated, duplicated mtDNAs were detectable using Southern hybridisation in blood, skeletal muscle, heart muscle, liver brain pancreas and ovary, but not bladder epithelial cells or hair. PCR revealed that they were present in every other tissue investigated, and although not strictly quantitative it is reasonable to suppose that the variation in quantity of product between different hairs and granulocyte clones may represent differences in the proportion of abnormal genomes in different clonal lines.

Surprisingly, the proportion of abnormal genomes was higher in blood than the first muscle sample in patient 1. However, it must be remembered that the muscle sample was obtained 9 years before the blood sample during which time her muscle power deteriorated. It may be that the proportion of duplicated genomes increases over time, but we have not yet analysed the remaining tissue samples densitometrically. Moreover, as only one muscle was biopsied it might not be representative of the overall distribution.

Determinants of the tissue distribution of abnormal genomes probably include the exact stage of development when the duplication occurred, and

factors influencing their propagation. This will be discussed further in the final discussion (chapter 10).

The location of the duplications is of interest because the nonduplicated or "deleted" region was similar to but larger than the deletions described in KSS or CPEO. Furthermore, duplications in a similar region have been described in lizards (Moritz and Brown 1987). This suggests that this region is probably prone to recombination, perhaps because of there are recombination "hotspots", but that perhaps different constraints determine the size of the deleted segment in the two different types of re-arrangent. For instance, it is likely that some of the deletions which occur have not yet been described because they are lethal or the defective genomes are unable to replicate.

The sequence data which has now been obtained from patients with deletions of mtDNA suggests a molecular mechanism for recombination. In many deletions there is a homologous portion in each strand at the breakpoint, ranging from 3-13 bp (for instance, in the so called "common deletion" there is an exact 13 bp homology, Fig 3.2 and 10.1). In neither of the duplications is there an extensive homology. In patient 2 there is a three bp homologous sequence in each strand at the break point. There is no homology at the breakpoint in patient 1. Other mechanisms which may play a part are discussed in chapter 10.

In conclusion, there is now good evidence that mutations in mitochondrial DNA cause disease in man. Many questions remain unanswered. Further work on rearrangements of mtDNA in health and disease will provide new insights into mitochondrial function.

Chapter 8: ResultsDetection of mitochondrial DNA deletions in blood using the polymerase chain reaction: non-invasive diagnosis of mitochondrial myopathy.

NB: Please refer to appendix 1 to cross reference patients appearing both in this chapter and chapter 8.

8.1 Summary

Southern hybridisation demonstrates deleted mitochondrial DNAs(mtDNAs) in muscle but not in blood in a subgroup of patients with mitochondrial myopathy. The polymerase chain reaction (PCR) was used to search for low levels of rearranged mitochondrial DNAs in blood in 24 patients with mitochondrial myopathy, and 15 asymptomatic relatives all of whom have no detectable abnormality on restriction enzyme analysis of blood mitochondrial DNA. In 8 patients PCR products were obtained consistent with deletions of mitochondrial DNA. The presence or absence of a deletion was correctly predicted in 10 out of 11 patients from whom information was available from muscle DNA. No false positives were obtained in 43 controls. PCR analysis of blood may be applicable as a non-invasive screening test of affected individuals and in carrier detections

In one family, two asymptomatic relatives also carried the deletion. This is evidence for the presence of deleted mtDNAsin the germline in these disorders, supported with sequence data. The patient carries a higher level of deleted mtDNAs than his relatives, corresponding to severity of symptoms, and consistent with a predicted dosage effect (Wallace et al. 1988b). "Selfishness" (Jacobs and Lonsdale 1987) of deleted mtDNAs is probably one of the factors over and above random segregation (the bottleneck hypothesis, Hauswirth and Laipis 1985) which may be invoked to explain the usual distribution of mtDNAs in different tissues of patients with mtDNA deletions.

8.2 Introduction

In patients with point mutations of mtDNA (Wallace et al. 1988a, Harding 1989b), with mtDNA duplications (chapter 7) and deletions associated with neonatal onset sideroblastic anaemia (section 3.4.2), the abnormal mtDNA is present in every tissue investigated so far. In these patients non-invasive diagnosis from a single blood sample is possible. In patients with mitochondrial myopathy and mtDNA deletions, different tissues show a wide variation in proportions of normal and deleted mtDNAs. Deletions which have been demonstrated by Southern hybridisation in muscle may also be apparent in

other tissues such as brain, liver and kidney (Lestienne et al. 1988, Moraes et al. 1989b). However, this technique is rarely able to detect them in blood (Holt et al. 1988b). Deleted genomes have recently been detected in blood using the polymerase chain reaction (PCR), at levels below the sensitivity of Southern hybridisation (Johns et al. 1989). We have now confirmed these findings with analysis of muscle samples and with sequence data, and suggest that non-invasive diagnosis and carrier detection may be possible in a proportion of patients.

8.3 Subjects

Blood DNA was analysed in 24 patients with mitochondrial myopathy (see table 8.1) and 43 controls (13 normals and 30 individuals known to be asymptomatic from a neurological point of view who had been enroled for an asthma study). Previous restriction enzyme analysis had not previously detected structural rearrangements in blood mtDNA from any of these individuals (but see below).

Clinical details of the 24 patients are summarised in Table 8.1. All patients had ultrastructural evidence of abnormal mitochondria on either light or electron microscopy except case 12 who was never biopsied and died of a glioma, and case 9 whose biopsy and cytochrome oxidase staining was normal. Both had clinical evidence of CPEO and mtDNA deletions.

Patient 1 presented with slurred speech and poor coordination at the age of 14. He developed weakness of his eye muscles, and limbs, retinal degeneration and heart block. Muscle biopsy revealed 11% ragged red fibres with 3% COX negative fibres and paracrystalline inclusions on electron microscopy. Southern blotting revealed 40% deleted mtDNAs in muscle (Holt 1989). Although initially Southern blotting of blood appeared to be normal (Chapter 6), once a deletion had been established in muscle, a similar band was just detectable in blood on over-exposing the autoradiographs, but not quantifiable with laser densitometry. The deletion in muscle mapped to the region of the so-called "common deletion" (Holt et al. 1989, Moraes et al. 1989, Schon et al. 1989, see Fig. 3.2) His asymptomatic mother also had 7% ragged red fibres and a few COX negative fibres, but no deletions were detected on Southern hybridisation of blood or muscle (Holt 1989).

CLINICAL DETAILS

Diagnosis	Patient No	Sex	FH	Biochemical defect	Deletion PCR on blood	Deletion Muscle blot
I. KSS	1[1]	M	*	-	Yes (cd)	Yes
	2	M	-	NA	No	Yes
CPEO	3[2]	M	-	I	No	NA
	4[3]	M	-	I & IV	Yes	Yes
	5[4]	M	-	NA	No	No
	6[4]	F	-	-	Yes (cd)	Yes
	7	M	-	NA	No	NA
	8	F	+	NA	Yes	NA
	9	M	-	NA	Yes (cd*)	Yes
	10	F	-	NA	Yes (cd)	NA
	11	M	-	NA	Yes (cd)	Yes
	12	F	-	NA	Yes (cd*)	NA
II	13[4]	F	+	I	No	No
	14[4]	F	-	I	No	NA
	15	M	+	NA	No	NA
	16	M	+	NA	No	NA
	17	F	+	I	No	NA
	18	M	+	NA	No	NA
III. MELAS	19[4]	F	-	NA	No	NA
	20[4]	F	-	I	No	No
	21	F	+	NA	No	NA
	22	F	+	NA	No	No
III. MERRF	23[5]	M	-	V & CN	No	NA
	24[4]	F	-	III & IV	No	No

Table 8.1: Clinical classification of patients, Roman numerals in the first column referring to the clinical categories of Petty et al. (1986), and in the fifth column to the respiratory complexes of the electron transport chain. Key: -: no abnormality detected. cd: common deletion NA: information not available CN: carnitine deficiency FH +: positive family history of likely mitochondrial defect *: deletion detected in asymptomatic family members (see text). *: PCR product sequenced directly. [1]see Holt (1989), [2] see Land et al. (1981), [3] see Johnson et al. (1983), [4] see Poulton et al. (1988), [5] see Petty et al. (1986)

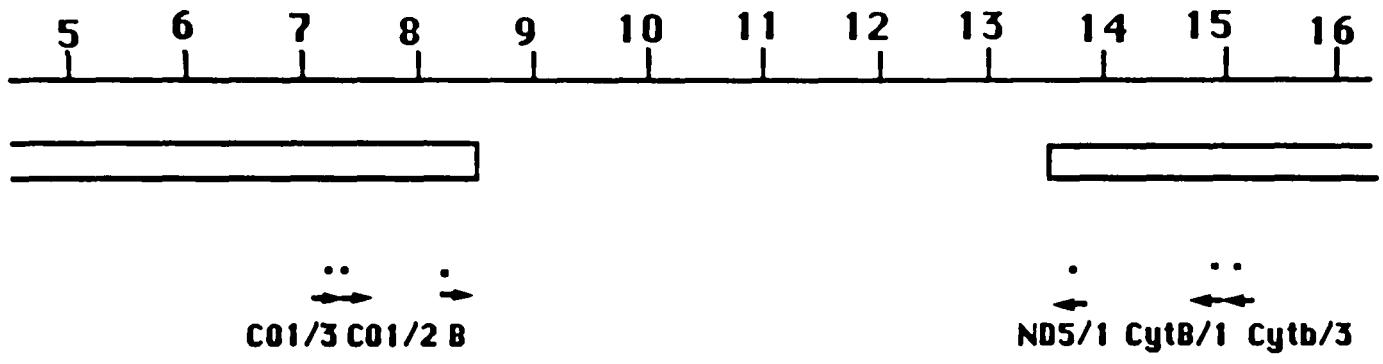


Fig 8.1

Position of 20mer oligonucleotide primers used relative to the published mtDNA sequence and position of the deletion. Upper scale: reference numbers in kilobases. Bars: mtDNA flanking the deletion. Dots: primer positions. Arrows: Primer direction. CO1/2, CO1/3, B, ND5/1, Cytb/1 and Cytb/3: primer names, corresponding to base numbers 7414 to 7433, 7193 to 7212, 8196 to 8215, and complementary to 13812 to 13831, 14955 to 14974 and 15132 to 15151

8.4 Methods

Southern Hybridisation: DNA was digested with BamHI to linearise both normal and abnormal genomes. This was electrophoresed in 0.8% agarose gels, Southern blotted, and probed with purified total mtDNA which had been labelled with ^{32}P-dCTP by random hexanucleotide priming.

Polymerase Chain Reaction: Oligonucleotide primers designed to be complementary to mt DNA sequences either side of the abnormal junction in mtDNA with the so-called "common deletion" (fig 3.2) were used under conditions described in table 5.3 (either primers CO1/2 and Cytb3 or CO1/3 and Cytb1 for 30 cycles, then 1/25 of the product was re-amplified with primers B and ND5/1 for 30 or 40 more cycles, see table 5.2,figs 5.2 and 8.1). The product was electrophoresed in 1- 1.5% agarose gels and stained with ethidium bromide.

Sequencing: PCR products were either sequenced directly using either primer ND5/1 or B (see 5.8.2) or cloned and sequenced (see 5.7 and 5.8.1)

8.5 Results

PCR: A PCR product of identical size (658 bp) was obtained in 6 patients, and a slightly smaller one in two further patients using primers CO1/2 and Cytb3 for 30 cycles, then primers B and ND5/1 (fig 8.2). No such product was obtained in any of the 43 controls. A second product of appropriate size was synthesized from these eight patients using another set of nested primers (Cytb1 and CO1/3 then ND5/1 and CO1/2).

Unaffected family members: PCR also revealed an identical product in patient 1, his mother and sib (in order of descending yield), although neither mother nor sib were symptomatic. A third round of PCR was performed from a non-deleted region in order to confirm that the starting quantity of DNA was not rate limiting (fig 8.3). These were sequenced and found to be the common deletion (two, three and one clone respectively). No product was obtained when a total of 13 other relatives of the other seven patients with confirmed or suspected mtDNA deletions were screened.

Southern Hybridisation of muscle mtDNA: Information from Southern hybridisation of muscle tissue was available in 11 patients. This included

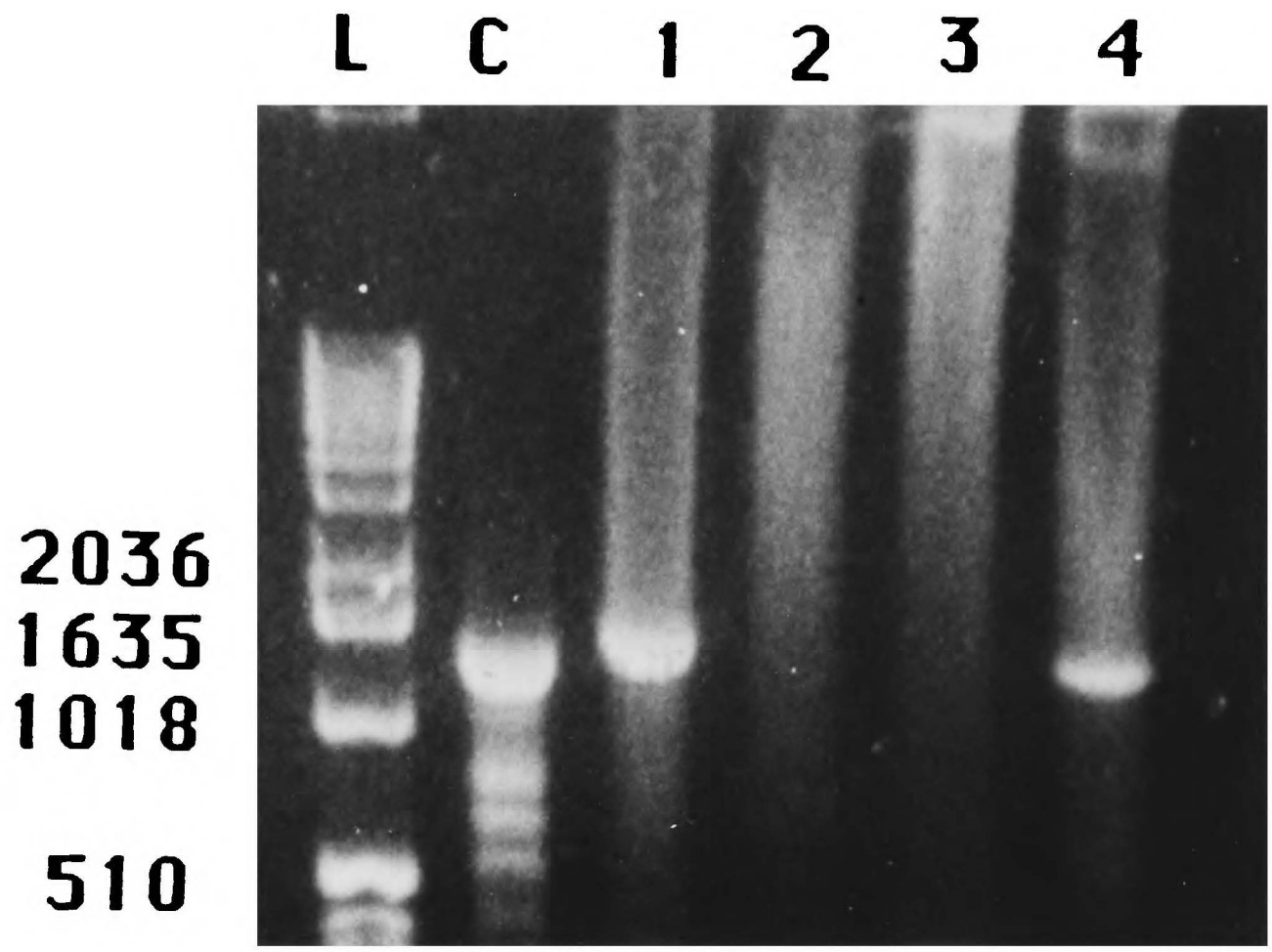


Fig 8.2

PCR products using nested PCR with primers CO1/3 and Cytb1 followed by CO1/2 and ND5/1 on blood, electrophoresed and stained with ethidium bromide. A product of approximately 1.5kb was obtained in patients 1 and 4 but not in patients 13 or 3 (or in 43 controls, results not shown).
L: size markers, C: PCR control 1-4: patients 1, 13, 3 and 4 respectively.

5 patients in whom PCR suggested a deletion, and confirmed that the predicted deletion was present in all five. Of the remaining 6 patients, only one had a deletion which had not been detected on PCR, and this mapped to a region which would not be detectable by the primers used.

Sequence analysis: Sequence analysis of the PCR product from blood was confirmed that the so called "common deletion" was present in 6 patients, and also 2 asymptomatic relatives of patient 1. In patient 4 a slightly larger deletion was present.

8.6 Discussion

8.6.1 The common deletion is detectable by this method

The polymerase chain reaction was able to detect low levels of abnormal mitochondrial genomes in blood of a substantial proportion of patients. In the patients in whom muscle data was also available, there were no false positives. If muscle had been available on all patients, deletions in different locations would probably have been detected in a proportion. However, it may be that most or all of the known deletions of mtDNA could be detected in blood using appropriate sets of primers. Most studies (Holt et al. 1988b, Moraes et al. 1989b) suggest that the common deletion comprises around half of deletions, compared to 6/9 here. The overall frequency of the common deletion in our patients (6/24 or 25%) is higher than expected from previous studies, probably because of the sampling differences and the relatively small numbers of patients investigated. However, it remains possible that other investigators have underestimated the proportion of patients with the common deletion by using Southern hybridisation rather than PCR. It is of particular interest that two of our deletion patients (cases 4 and 9) did not have classical histological appearances of mitochondrial myopathy on the initial biopsy. In one case these were apparent on a second later biopsy. Analysis of mitochondrial DNA was thus more sensitive than classical histology in these two patients. Furthermore, it is possible that subclinically affected individuals such as the two assymptomatic relatives of patient 1 might have levels of deleted mtDNAs below the sensitivity of Southern hybridisation in all tissues. If so, the use of PCR might increase the proportion of patients with MM in whom deletions are detectable.

8.6.2 Reliability

As no product was detectable in any of the 43 controls, the false positive rate is likely to be low. This is extremely important as it is well known that if conditions are not properly controlled "false priming" (eg of nuclear sequences) may occur. Although this possibility has been excluded here by sequencing, this is unlikely to be practicable if used by routine laboratories as a screening test. In this case, the mitochondrial origin of products can be confirmed by restriction mapping or Southern hybridisation. For example, spurious products may be obtained if the annealing temperature is too high, the extension time too long, the starting concentrations of DNA, enzyme or primer are too high, or the magnesium concentration is not optimised. Furthermore, the use of nested sets of primers in 2 serial reactions (eg. Cytb1-Co1/3 then ND5/2-B) increases the specificity of PCR. Primer specificity is a function of length. Our primers are designed on the basis of a homology search to minimise the likelihood of nonspecific priming of mtDNA. As it stands our protocol appears to be reliable, but we advise against modifications using alternative primers unless extensive controls are run.

8.6.3 Applications

This approach has two clear applications: it may enable non-invasive diagnosis in up to 40% of patients with mitochondrial myopathy, and might be useful in genetic counselling. Muscle biopsy is an invasive procedure, and may impair mobility in an already weak individual. Although PCR on blood is unable to differentiate between deletions and duplications, it could be diagnostic by identifying an identical junction fragment in families where one member has already been biopsied, or useful as an initial screen in patients where MM is suspected. Furthermore, it was more sensitive than routine histology in identifying a mitochondrial defect in one case, although this would have been detectable on Southern hybridisation of muscle mtDNA.

Secondly, this technique might be useful to detect individuals at risk for transmitting the disease. In family 1, identical deletion sequences were present in the blood of proband and two assymptomatic relatives. Both of these two individuals must therefore be at risk of developing the disease,

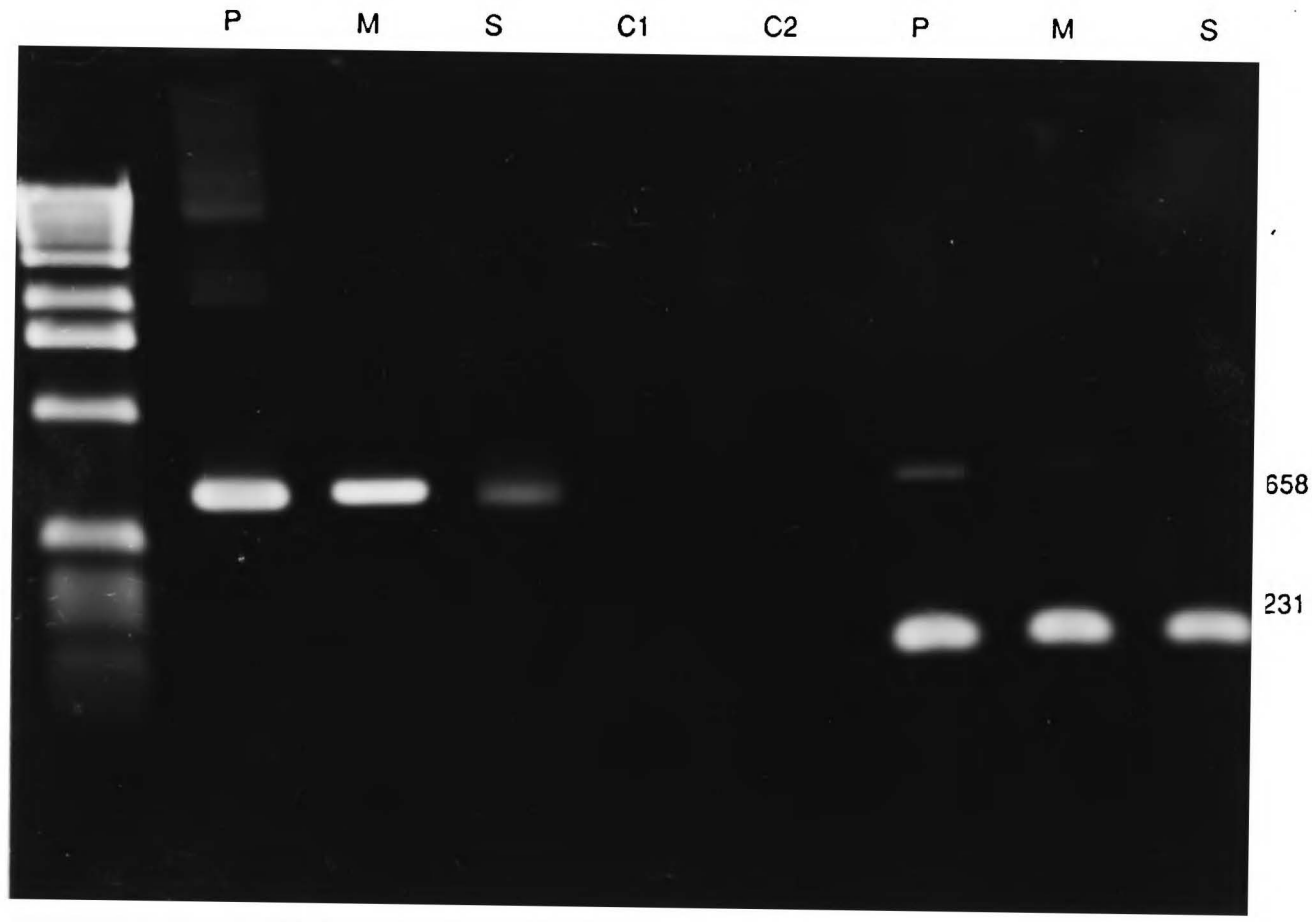


Fig 8.3

PCR products electrophoresed in 1.5% agarose gels and stained with ethidium bromide in family 1.

Lane 1: size markers. lanes 2-6: products using primers CO1/2 and Cytb3 for 30 cycles, then re-amplifying 1/25 of the product with primers B and ND5/1 for 40 more cycles. A 658bp product was obtained in the patient, mother and sib but not in controls. (KEY: P: patient, M: mother, S: sister, C1 and C2: controls). Lanes 7-9: In order to confirm that differences in yield were not due to insufficient starting DNA, 1/25 of the final reaction mixture for patient, mother and sister was re-amplified with control primers ND4/6 and 7

and of transmitting it to future offspring. Further studies are needed to assess its predictive value in these cases before it can be used to advise patients.

8.6.4. Germline deletion of mtDNA in family 1 (fig 8.3)

Although it is theoretically possible that the six clones sequenced for family 1 represented only one out of a family of rearrangements of mtDNA (Zeviani et al. 1989a), it must be the predominant population as there was no evidence of others on Southern analysis or PCR. The most likely explanation is that this deletion did not occur three times independently, but is present in the germline, and that deleted mtDNAs have been passively transmitted from mother to both her offspring. Although very accurate quantitation is not possible, symptoms correlate with the proportion of deleted mtDNAs. Both Southern hybridisation and PCR demonstrated that there were more deleted mtDNAs in blood and muscle (Holt 1989) of the proband than his mother. There are no published examples of this dosage effect between individuals with the same proven mutation (Moraes et al. 1989b), although increasing levels of deleted mtDNAs have been reported in the same deteriorating individual (Larsson et al. 1990). It is likely that symptoms depend on many factors, such as the distribution of deleted mtDNAs within a cell and within a tissue as well as the energy demands on the tissue. This will be discussed further in the Chapter 10.

Finally, there is a marked similarity between the distribution of the tissues affected in this patient and the distribution in other deletion patients, with much higher levels of deleted genomes present in muscle than blood. In these other sporadic cases, the explanation could have been the occurrence of somatic mutations in muscle. However, as the phenotype and tissue distribution of this germline mutation is similar to those of the majority of reports, it is most unlikely this is merely the result of the "bottleneck" followed by random segregation as described above: other factors may also be operating.

8.7 Conclusion

In conclusion, the polymerase chain reaction was able to detect low levels of abnormal mitochondrial genomes in blood of a substantial proportion of patients with mitochondrial myopathy, and may ultimately be useful for noninvasive diagnosis and genetic counselling.

Chapter 9: Results

Analysis of Mitochondrial DNA in Leber's Hereditary Optic Neuropathy

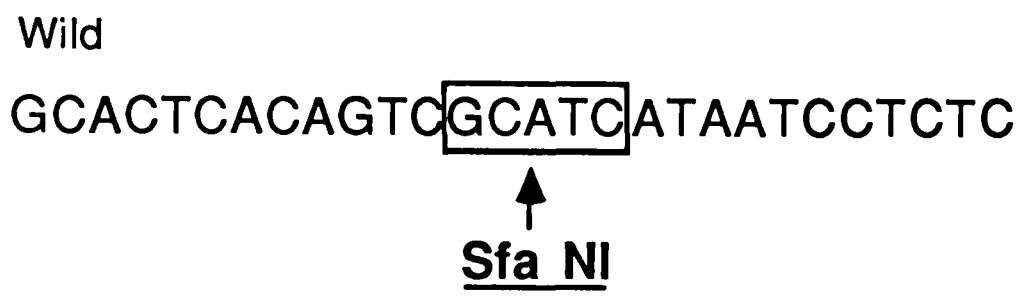
9.1 Summary

19 patients with Leber's Hereditary Optic Neuropathy (LHON) were investigated for the presence or absence of the point mutation described by Wallace et al., using Southern hybridisation, and sequencing, oligonucleotide probing and competitive oligonucleotide priming of PCR products. In 14 cases the point mutation was confirmed, and 5 had the wild-type sequence in this region. Of the 5 patients with the normal sequence, only one had a clear family history of LHON. Sequence analysis in this patient revealed a single base insertion between bp 11,718 and 11,719 producing a frame shift. If transcribed and translated this would result in a truncated peptide.

This provides evidence that Wallace's mutation is present in the majority of patients with LHON, and support for the view that both mutations are causative.

9.2 Introduction

The maternal pattern of inheritance in Leber's Hereditary Optic Neuropathy (LHON) led to suggestions that it might be caused by a mutation in mitochondrial DNA (mtDNA), because mtDNA is exclusively maternally inherited. While early restriction enzyme analysis of blood mtDNA revealed neither a major rearrangements, nor an association with a particular mtDNA type (Holt et al 1989b, Vilkki et al. 1989a), a much more detailed study identified a point mutation in mtDNA (Wallace et al. 1988a). Indirect evidence suggests that it is causative, but no definite mechanism has yet been identified. Not only is the mutation present in the majority of LHON families (Wallace et al. 1988a, Vilkki et al. 1989b), but it has been confirmed in several different ethnic groups (Ozawa et al. 1989, Singh et al. 1989) and comparison of mtDNA typing suggests that it occurred independently at least twice in the past 40-80,000 years. This mutation is an A to G transition at base pair number 11778, and results in the replacement of a conserved arginine residue with a histidine (fig 9.1). It can be detected by Southern hybridisation because it causes an SfanI site loss. While some families appear to be homoplasmic for this defect, others are heteroplasmic, severity correlating with dosage of abnormal mtDNAs (Holt et al. 1989b).



Legend to fig 9.1:
Mitochondrial DNA sequence in the region of MTND4 showing the G to A transition at bp 11,778 found to be present in the majority of patients with LHON resulting in the replacement of a conserved arginine residue with a histidine.

Table 9.1

Patient details

Patient no.	Sex	Family history?	Sequence at bp 11,778
Leber's hereditary optic neuropathy			
1	M	+	L
2	M	+	L
3	M	+	L
4	M	+	L
5	M	+	L
6	M	+	L
9	M	-	W
11	M	+	L
12	M	+	L
13	M	+	L
14	M	+	L
15	M	+	L
16	M	-	L
17	F	+	L
18	M	+	L
19*	M	-	W
20	F	-	W
21	M	+	W@
22	M	-	W
Autosomal dominant optic atrophy			
7	M	+	W
8	M	+	W
11	F	+	W

Key: + : other affected family members consistent with diagnosis.
- : sporadic
L : 11,778 mutation present
W : normal sequence at bp 11,778.
* : blood sample from asymptomatic mother not from proband
@ : Point mutation in MTND4 at bp 11718-9.

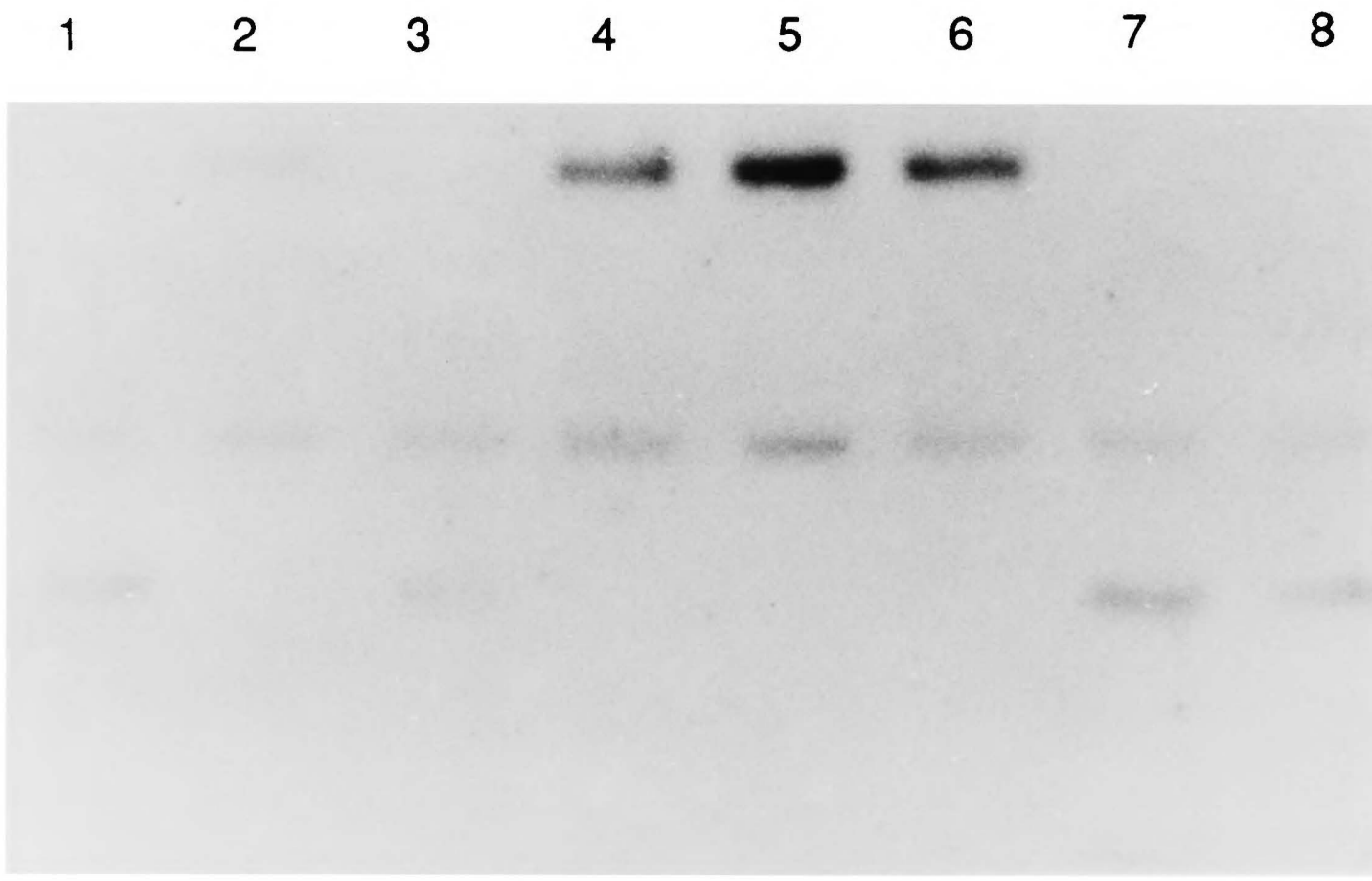
We have studied 19 with patients with LHON and 12 controls who included 3 patients with dominantly inherited optic neuropathy and 9 normally sighted individuals. Southern hybridisation, sequence analysis, oligonucleotide probing, competitive oligonucleotide priming (COP, see section 5.5.6) of PCR products of blood DNA were used to detect the presence of this point mutation. These last three methods were used in addition to Southern hybridisation, to obtain more information than is apparent from the loss of a restriction enzyme site. Sequence analysis was performed on blood from LHON patients in whom the Sfa Nl site was intact in order to ascertain whether there was an alteration in the first base position of the arginine codon which would not intersect the enzyme's recognition sequence. Oligonucleotide probing was performed in order to confirm that the site loss was due to the same base change in all patients and to confirm the sequence data. COP was used in an attempt to investigate heteroplasmy, as it is potentially more sensitive at detecting minority populations than the other methods.

9.3 Patients.

These were diagnosed at the Tennant Institute of ophthalmology, Glasgow, by Dr Bronte-Stuart and Professor Foulds (21 cases) and at the Oxford Eye Hospital by Prof Bron (1 case). Blood was obtained from affected individuals (21 cases) or unaffected mother who was a possible carrier (1 case). Clinical details are summarised in Table 9.1. No patients with LHON were known to be related, although there was a father-son pair among the patients with dominantly inherited optic atrophy. Diagnostic criteria for LHON were acute painless onset of visual impairment becoming progressively severe over a period of days, weeks or months, with the appearance of optic atrophy in the absence of other causes, becoming bilateral within 9 months. As there is no absolute diagnostic feature, only patients considered typical or with a clear family history were included. In contrast, patients with dominantly inherited optic atrophy (dominant OA) had moderate or mild non-progressive visual impairment and autosomal dominant pattern of inheritance.

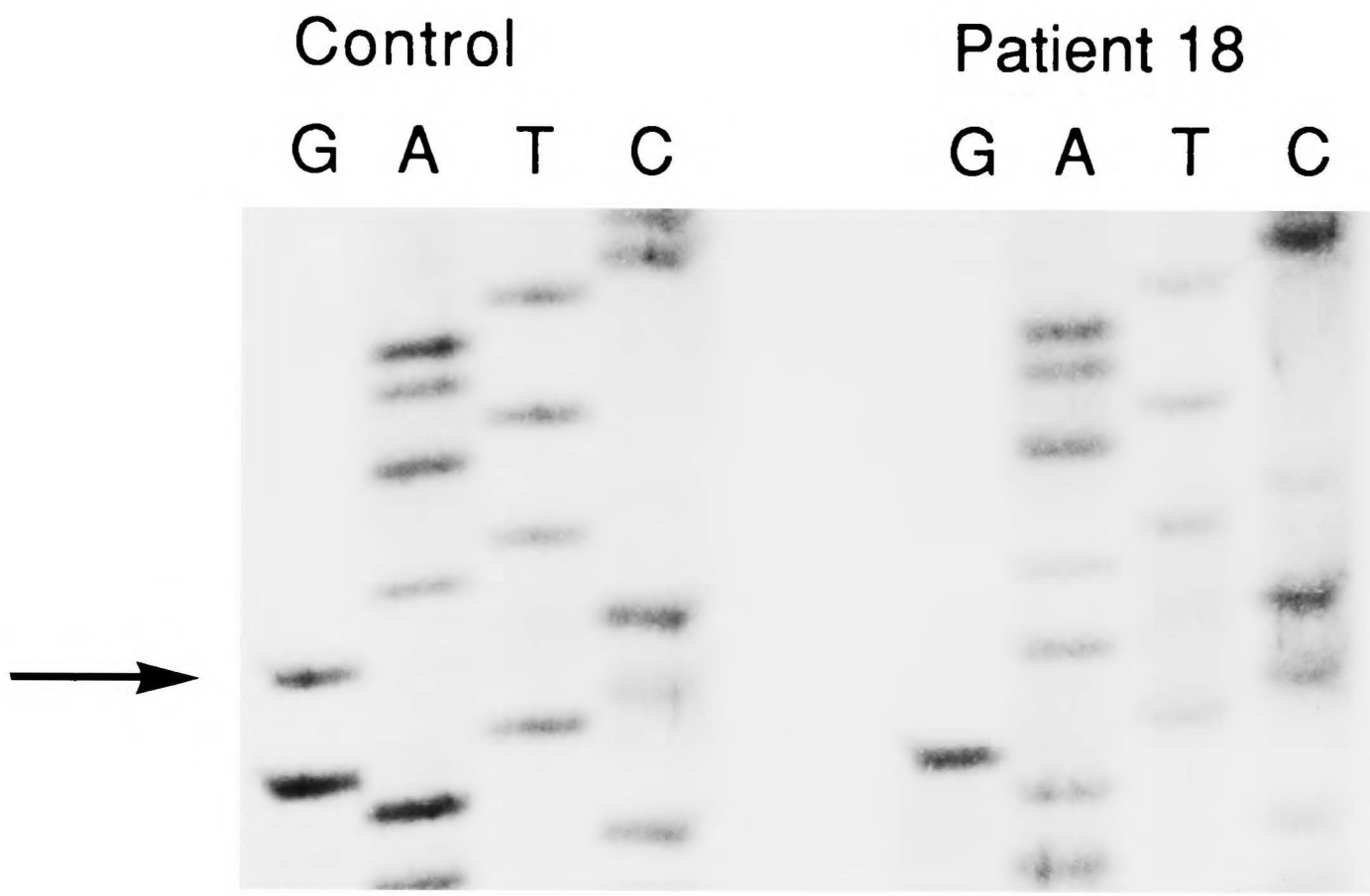
9.4 Southern hybridisation

Blood DNA was digested with Sfa Nl, electrophoresed on 1.5% gels, Southern blotted and probed with regional probe 11 (Fig 5.1). Loss of the Sfa Nl site resulted in the replacement of the 915 and 679 bands with a



Legend to Fig 9.2

Blood DNA was digested with Sfa Nl, electrophoresed in 1.5% agarose gels, Southern blotted and probed with regional probe 11 (see fig 5.1). Loss of the restriction site resulted in the replacement of the 915 and 679 bands with a 1.594 kb band. Lanes 1-8 patients 22, 18, 19, 17, 16, 15, 21,20. Control (not shown) was identical with lanes 1, 3, 7 and 8.



Legend to fig 9.3

Sequence analysis of the PCR product ND4/6-7 by the unbalanced priming method. The G residue at bp 11778 is replaced by A in the mutant in patient 18 but not the control.

Ala His Glu Leu Thr Ser Ser Leu Leu Phe Cys Leu Ala Asn Ser Asn Tyr

GCCCACGGGCTTACATCCTCATTACTATTCTGCCTACGAAACTCAAACTAC

Wild

Glu Arg Thr His Ser Arg Iso Met Iso Leu Ser Gln Glu Leu Gln Thr Leu

GAACGCACTCACAGTCGCATCATAATCCTCTCTCAAGGACTTCAAACTCTA

Ala His Glu Ala Tyr Ile Leu Ile Thr Ile Leu Pro Ser Lys Leu Lys Leu

GCCCACGGAGCTTACATCCTCATTACTATTCTGCCTACGAAACTCAAACTA

Patient 21

Arg Thr His Ser Gln Ser His His Asn Pro Leu Ser * * *

CGAACGCACTCACAGTCGCATCATAATCCTCTCTCAAGGACTTCAAACTCT

Legend to fig 9.4
Sequence analysis of the PCR product ND4/6-7 in patient 21. There is an insertion of an a residue between bp 11718 and 11719, which generates a "stop" codon and hence trucated peptide. The conserved arginine residue (fig 9.1) is once again replaced, and there are two histidine residues immediately adjoining the position corresponding to the 11778 mutation.

1.594 kb band in 14/19 individuals (fig 9.1, 9.2 and table 9.2). No evidence of heteroplasmy was seen, although partial digests were obtained on a number of occasions.

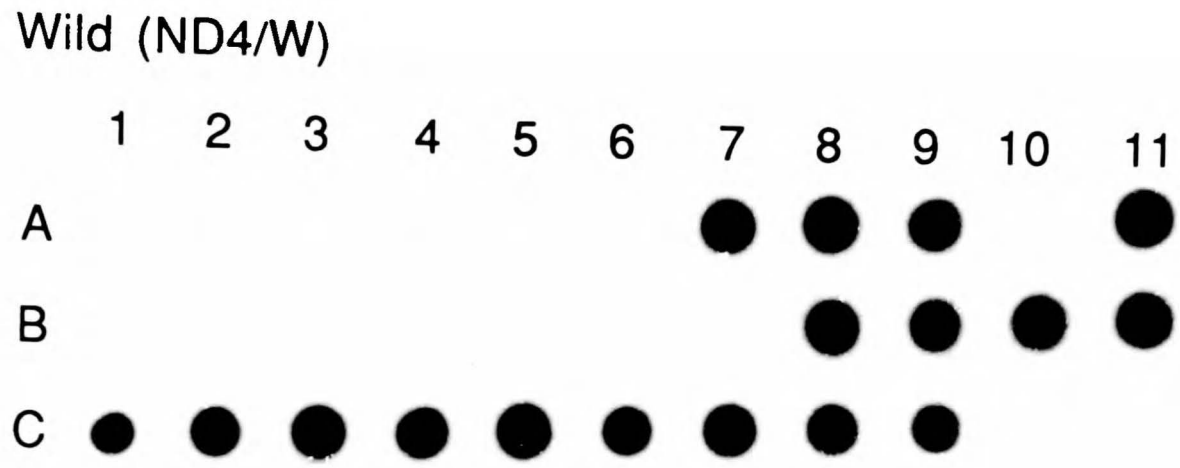
9.5 Sequence analysis

PCR was used to amplify a 231 bp region of MTND4 (with primers ND4/6 and ND4/7) from patients and controls for this and the remaining analyses (fig 5.2). In two patients (numbers 20 and 21) the PCR product was cloned and sequenced (as in section 5.7). Direct sequencing of PCR products was also performed in these and the other 2 LHON patients and 1 mother with an intact Sfa Nl site, ie individuals 9, 19, and 22 (sections 5.5.4 and 5.8.1). Primers were either ND4/1 and ND4/3 or ND4/7 and ND4/7 followed by unbalanced priming with ND4/6 and 7 (an excess of ND4/6 followed by sequencing with ND4/7 or an excess of ND4/7 followed by sequencing with ND4/6). Sequence analysis was also performed on the following patients: 15, 16, 17, 18 (fig 9.3).

In all cases the results confirmed the data shown in table 9.2. Normal sequence in this region was obtained in all individuals in whom the Sfa Nl site was intact. In particular, there were no alterations in the first base position of the arginine codon which would not interrupt the enzyme's recognition sequence. However, a significant mutation was identified in patient 21 who had a normal Sfa Nl restriction pattern and familial LHON, 60 bp upstream: a single base insertion between bp 11,718 and 11,719 produced a frame shift. If transcribed and translated this would result in a truncated ND4 polypeptide, consisting of the first 320 residues of normal sequence, followed by 26 abnormal residues, instead of the normal 459 residues. It may be noteworthy that the conserved arginine residue is once again replaced, and there are two histidine residues immediately adjoining the position corresponding to the 11778 mutation (see fig 9.4)

9.6 Oligonucleotide probing

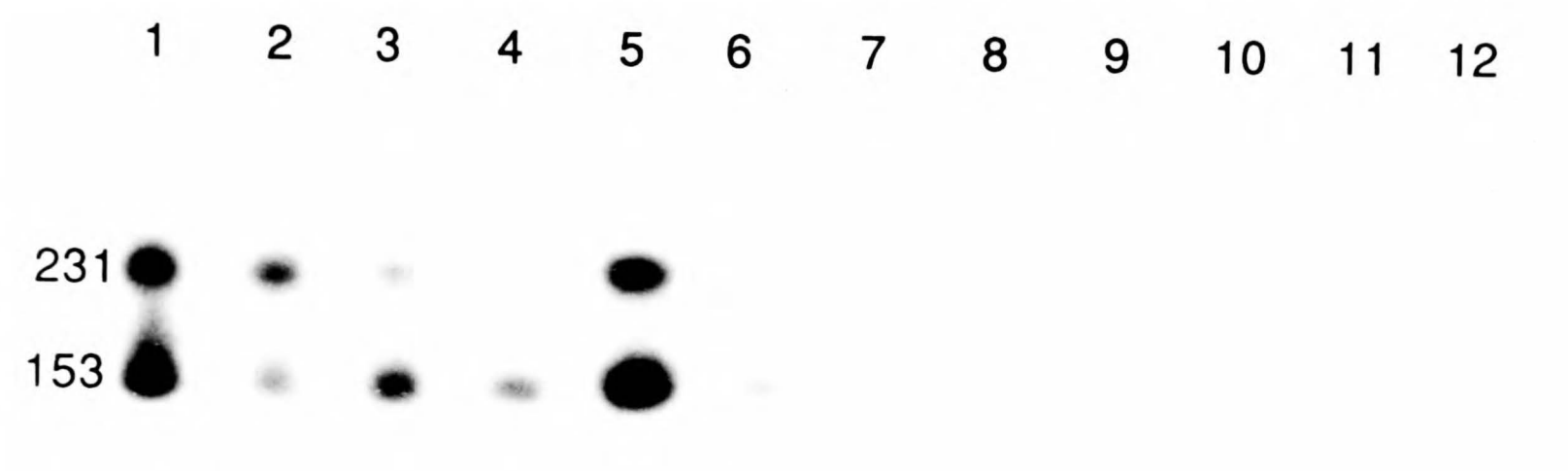
The advantage of this technique over Southern hybridisation is that it specifically detects the exact mutation. In this case, approximately 200ng of the same 231bp PCR product described above was dot blotted (section 5.6). This filter was probed with allele-specific oligonucleotides (section 5.2.4) which had been radiolabelled with gamma ^{32}P-ATP (ND4/W and ND4/L corresponding to the wild type and the mutated sequence, section 5.4.2, table 5.2 and figs



Legend to Fig 9.5

Dot blot filter of PCR product ND4/6-ND4/7 probed with end-labelled ND4/W (or the oligonucleotide corresponding to the wild type sequence, upper photograph) and ND4/L (or the oligonucleotide corresponding to the mutated sequence, lower photograph)

Row A, columns 1-11: patients 1-11. Row B, columns 1-11: patients 12-22. Row C columns 1-9: controls



Legend to Fig 9.6

Competitive oligonucleotide priming.

Lanes 1 and 7 : patient 15. Lanes 2 and 8, 3 and 9: controls. Lanes 4 and 10 : patient 21. Lanes 5 and 11 : patient 20 Lanes 6 and 12 : patient 19 (loaded in this order to minimise confusion from overflow from one well to the next). The oligonucleotide specific for the 11,778 mutation was end-labelled in lanes 1 and 8-12, the wild-type oligonucleotide was end-labelled in lanes 2-7.

5.2 and 9.5). The signal was positive with either one or the other probe, providing no evidence of heteroplasmy. Further, it confirmed that the site loss was due to the G to A transition in the 14/19 LHON patients (table 9.2). It also confirmed that the wild-type sequence was present in the remaining 4LHON patients and 1 mother, and the controls.

9.7 Competitive oligonucleotide priming

This result was further confirmed using Competitive oligonucleotide priming. This is similar in principle to oligonucleotide probing, only it uses PCR to detect hybridisation (Gibbs et al. 1987). Both allele-specific primers are present in the reaction along with an appropriately placed oligonucleotide for priming synthesis of the complementary strand. The alternative primers compete with each other but the exact match primes about 100 times more often. This can be demonstrated by labelling one or other of the primers and investigating incorporation of radioactivity into the product by electrophoresis (fig 9.6). Primers ND4/W and ND4/L were used with primer ND4/6. Once again the results confirmed those shown in table 9.2: radiolabelled primers were only incorporated in reactions where they exactly corresponded to the template sequence when 20 cycles were performed using annealing temperatures of 48°C or above were used. At lower temperatures (below 43.5 and 46.5°C for ND4/W and ND4/L respectively) non-specific priming occurred: incorporation into PCR product using cloned wild-type template was similar to that obtained when the template was also contaminated with 1% of DNA from a mutant LHON product and vice versa. No consistent results suggesting heteroplasmy were obtained in either group: that is, the results of Holt et al. (1989b) were not confirmed: there was no evidence that the LHON patients with wild-type sequence also carried low levels of mutant mtDNA.

9.8 Discussion

All four methods confirmed that 14 out of the 19 patients with LHON had the expected point mutation, but not the 3 patients with dominant OA or the 9 controls. Sequence data on cloned DNA from two patients of the five patients without the site loss excluded other substitutions of the same amino acid. Similarly, direct sequencing of the remaining 3 patients in this group again revealed wild-type sequence around bp 11,778, (although in one case a significant mutation was identified 60 bp upstream). This data provides

Table 9.2

Incidence of point mutation in bp 11,778

	Normal n (%)	mutation n (%)	TOTAL
LHON	5 (26)	14 (74)	19
Dominant OA	3 (100)	0 (0)	3
Controls	9 (100)	0 (0)	9

Table 9.3

Patients Studied

	Familial	Sporadic	TOTAL
LHON: with 11,778 mutation	13	1	14
wild type at 11,778	1	4	5
Dominant OA	3	0	3
TOTAL	17	5	22

further evidence that the mutation at base pair 11778 is present in the majority of patients with LHON. In particular, the finding of a point mutation which causes a frame-shift in the patient with familial LHON but no Sfa Nl site loss gives strong support to the hypothesis that either of these two point mutations in MTND4 may be causative. However, until there is more information about the likely mechanism and more control data for both these loci, this view is contestable.

None of the methods used provided support for the view of Holt et al (1989b) that heteroplasmy is present in a proportion of affected individuals with the 11,778 mutation, and that there is a dosage effect, even though this has now been confirmed by Wallace (1990). There are a number of possible reasons for this discrepancy. Firstly, the levels of normal mtDNAs in this group of 14 individuals may be extremely low: this group was confined to affected individuals, and no asymptomatic relatives who would be expected to carry a higher level of normal mitochondrial genomes in blood. Secondly, only patients who very clearly fulfilled the diagnostic criteria were included in this series: it is possible that if a wider group of patients had been included that the levels of normal mtDNA might have been higher. In support of this, the proportion of patients with the 11,778 mutation was higher in this series than has been found in two recent studies (Holt et al. 1989b and Vilkki et al. 1989), suggesting that there may be differences in patient population and/or diagnostic criteria between centres. Thirdly, none of the techniques used was especially sensitive for detecting low levels of normal mtDNAs. Southern hybridisation can detect levels down to about 5% and oligonucleotide probing is less sensitive than this. Sequence analysis of a single clone is likely to reflect the major population of mtDNA, and it would be necessary to sequence 15 clones in order to be more than 80% certain of detecting a minority population of less than 10% of mtDNAs. Direct sequencing of PCR products has been used to detect heterozygosity of nuclear genes, but in practice "ghosting" prevents detection of low levels of abnormal genomes with certainty on a single run. It was anticipated that competitive oligonucleotide priming would be able to detect low levels of minority populations of mtDNA. In practice, under conditions where the minority population of mtDNA in a 99:1 mix gave a signal, there was considerable non-specific priming for both primers. The level of this non-specific background depended on total yield of PCR product. The variation in

yield between different templates in the same reaction resulted in varying backgrounds and precluded the identification of heteroplasmy. This might be simply a property of the primers used, and it is likely that other techniques based on PCR would be effective.

There are several possible explanations for the identification of 4 families in whom the major population of mtDNA has the wild type sequence in this region. In one case, blood was only available from the proband's asymptomatic mother who would not carry the site change if a new mutation had occurred in the proband. None of these patients had a definite family history (table 9.3), and it is therefore possible that they carry a nuclear rather than a mitochondrial mutation. Alternatively, they might exhibit heteroplasmy with a wide variation in genotypic ratio between tissues: there might have been a somatic mutation in the developing eye at bp 11,778 which is not detectable in blood. Unfortunately this hypothesis is difficult to test, as appropriate pathological specimens are rare because patients with LHON die from other causes. Finally, it is possible that there are other mitochondrial point mutations, and for this reason further sequence analysis is in progress.

9.9 Conclusion

This data provides further evidence that the mutation at base pair 11778 is present in the majority of patients with LHON. The new mutation in MTND4 in patient 21 provides support for the hypothesis that either of these two mutations may be causative and give rise to the maternal pattern on inheritance. However until a mechanism has been identified and more control data obtained, this is still open to question.

Chapter 10: Discussion

10.1 Introduction

The work described in this thesis raises many new questions. This final discussion section will attempt to cover some of these in more detail. Firstly, what is known or can be inferred about the possible mechanisms whereby these mutations of mtDNA give rise to the phenotype? Secondly, how did the mutations arise and can the variation in tissue distribution be explained? Thirdly, can this work be applied to clinical genetics with a view to non-invasive diagnosis of affected individuals and asymptomatic relatives, and possible antenatal diagnosis? Finally, what directions remain to be explored?

10.2 Pathophysiological mechanisms in mtDNA deletions.

The possible mechanisms by which mtDNA deletions (section 3.4.1) impair mitochondrial function will be discussed first, as much more is known about mtDNA deletions than mtDNA duplications, and the two may well be related. There are at least two ways in which deleted mtDNAs could result in deficiencies in protein subunits of the electron transport chain. Firstly, in all but one (unconfirmed) case (Saifuddin Noer et al. 1988), the deletions include at least one tRNA. As mitochondria have their own genetic code and mechanism for protein assembly, it is likely that organelles containing only deleted mtDNAs would be non-functional. This might be a minor problem if they were able to take up normal genomes (exchange of DNA between mitochondria is described in plants, Kemble et al. 1988), fuse with other mitochondria or were able to take up exogenous tRNAs. However, it might be argued that cytoplasmic tRNAs would be of limited use, as they do not use the same genetic code. Heteroplasmic mitochondria, or those able to take up sufficient tRNAs because they are located in a cytoplasm containing a high proportion of normal mtDNAs (complementation) might be only slightly impaired. The second hypothesis is that deficiencies arise because of a reduction in the product of one of the deleted protein reading frames, or production of an abnormal product from the junction region. The first hypothesis predicts that the biochemical defect should be similar in all deletions including a number of tRNAs, the second that the biochemical defect

should depend no the region of mtDNA which has been deleted. Several authors have commented on the apparent lack of correlation between location of the deletion and biochemical defect (Holt et al. 1989c, Moraes et al. 1989b). Furthermore, if the first hypothesis is correct, then the deleted genomes should be transcribed but not translated: if the second then both should occur. Northern analysis (a technique for analyzing RNA) has revealed an abnormal transcript, but so far no abnormal peptides have been detected (Nakase et al. 1990). Similarly, in situ hybridisation (a technique for probing tissue samples with DNA or RNA probes) has shown that "ragged red fibres" (which are not all red: individual fibres may change from normal to ragged red along their length (Mita et al. 1989b) contain low levels of mRNA from the deleted region, but normal or high levels from the non-deleted region. The technique has also shown that these fibres are deficient not only in peptides encoded by the deleted region, but also in mitochondrially encoded peptides from the nondeleted region. Nuclear encoded subunits are present, as is transcribed RNA (Mita et al. 1989b). In view of these data the first hypothesis has the most immediate appeal. However, very recently Szymkowiak et al. (1990) have evidence that at least some of the mitochondrial tRNAs are transported into the mitochondrion from the cell cytoplasm (cf section 3.2.3). They were able to detect two mitochondrial $tRNA^{meth}$ species, suggesting that there was either post transcriptional modification or uptake of cytoplasmic tRNAs. Thus, the plausible hypothesis that mtDNA deletions cause their phenotypic effects as a consequence of tRNA deficiency is less secure.

In all cases of MM with mtDNA deletions described so far, the tissue distribution is similar in outline: the highest proportion is found in muscle, but deleted DNAs may be found at variable levels in other tissues (section 3.4.1). Similarly, deleted genomes appear to be distributed more widely in KSS than CPEO, consistent with their clinical involvement. Thus, there may be a dosage effect: clinical severity increases with increasing proportion of deleted genomes within an individual or tissue (Larsson et al. 1990). It also seems likely that different tissues may have different thresholds for levels of deleted genomes required before being clinically involved, depending on their energy demands and developmental stage (Wallace et al. 1988b). However, careful comparisons of levels of deleted mtDNAs in patients with identical deletions does

not reveal a clear relationship between dose of deleted mtDNAs and effect between individuals. There are at least two possible reasons for this. Firstly, most of the discussion ranges around data on proportions rather than absolute levels: if deleted genomes are nonfunctional, then their clinical effect could act purely as a relative deficiency in normal mitochondria. Secondly, the distribution of normal and abnormal genomes within cells and organelles may determine the degree of complementation which occurs. Moraes et al. (1989a) have shown that single cells may be heteroplasmic (ie contain both normal and deleted mtDNAs) and that the ratio of the two types of mitochondrial genomes may vary from cell to cell within a tissue. It is possible that deficiencies in transcripts from the deleted regions are less likely to be rate limiting if there are normal mtDNA molecules as well as deleted ones in the same mitochondrion. Alternatively, complementation may occur due to uptake of tRNAs into mitochondria (as occurs in other organisms, see section 3.2.1) containing only deleted mtDNAs. Neither of these arguments addresses the question of how the characteristic distribution of deleted mtDNAs arises, and this will be discussed in more detail in section 10.5.

Presuming that some of these explanations will ultimately turn out to be correct, what is the explanation for the phenotypic differences in age of onset, severity and tissue involvement in Pearson's syndrome (section 3.4.2) and mitochondrial myopathy in the presence of apparently identical mutations? Clearly the difference in the tissue distribution of the abnormal genomes may be responsible for the differences in the tissues which are clinically affected. Secondly, many of the enzymes of the electron transport chain have both tissue specific and developmentally regulated isoforms, which might differ in their interactions with the mitochondrially encoded subunits. In general, the systems manifesting severe symptoms corresponded to the tissue distribution and dose of deleted genomes. Thus the threshold "dose" (Wallace et al. 1988b) of abnormal genomes required to produce a detectable effect in each tissue might be age-related.

10.3 Pathophysiological mechanisms in mtDNA duplications.

It is not clear how duplications could give rise to phenotypic effects, as apparently "silent" duplications in a similar region have been demonstrated

in lizards (Moritz and Brown 1987). Although it is possible that an excess of normal products deranges mitochondrial function (for example excess normal subunits of complex I could unbalance the kinetics of multimer assembly), it is more likely that products of the fusion gene cause problems by competing with the normal ones. There is a precedent for the latter in plants (Young and Hanson 1987). We have shown that the junction regions of both the duplications are transcribed, and if translated would result in truncated cytochrome oxidase subunit I peptides. Although it is not possible to predict exactly how these peptides would impair enzyme activity, it is noteworthy that this is the major catalytic peptide of complex IV. Alternatively, transcription products from the abnormal region might compete at the level of mRNA processing or translation. This hypothesis has some support, as Attardi (1989) has shown that control of mitochondrial protein synthesis probably occurs at the translational rather than the transcriptional level.

On the other hand, the preliminary finding of high molecular weight forms comprising repeats of the duplicated segment (chapter 7) suggests an alternative mechanism. It is conceivable that these molecules, which appear to be effectively multimers of deleted genomes, comprise the only mtDNA type in some mitochondria, or a high proportion of mtDNA in some cells and impair mitochondrial function in the same way as mtDNA deletions (for instance, tRNA deficiency, section 10.2).

10.4 Pathophysiological mechanisms in LHON.

The mechanism whereby the point mutation described by Wallace et al. (1988a) impairs mitochondrial function remains unclear. As very little is known about the active sites in this or any other subunit of complex 1, the effect of an arginine to histidine substitution cannot be predicted. However, the point mutation described in chapter 9 in patient 21 provides support for its significance. If the frame shift (and hence truncated peptide) is confirmed, it would be strong evidence in its favour. So far, complex 1 deficiency is not an established feature of typical LHON, although there is no data about the activities of the respiratory enzymes in patients' optic nerves. It is remains possible that the amino acid substitution in the ND4 subunit alters an interaction with a tissue-specific isoform resulting in a local complex 1 deficiency. In any case, both the preponderance of affected males and the age

of onset (section 3.4.3) require some kind of interaction with a nuclear gene product. It is noteworthy that respiratory defects have been difficult to document in CMS despite the overwhelming evidence that the mtDNA mutations are causative.

10.5 Origins of and determinants of tissue distribution of mtDNA rearrangements.

Despite the progress that has been made in characterising rearrangements of mtDNA in mitochondrial myopathy, many questions remain. The origins, inheritance and factors affecting tissue distribution of the abnormal genomes are not clear. These issues will be considered in turn.

Most of the discussion about the origins of mtDNA rearrangements in MM has centred on the homologies which have been observed at either end of deleted segments (Schon et al. 1989a, Holt et al. 1989a, Zeviani et al. 1989a). It is well known that homologies of this type may predispose to recombination events in other systems (Anderson and Roth 1977) such as bacterial genomes. However, as mentioned above, recombination is a rarity in mitochondria, and many of the mechanisms hypothesized for recombination in bacteria are probably not appropriate for mtDNA. However, the most popular hypothesis is that deletions occur during replication. The replication fork has to proceed from the origin of replication for the heavy strand round 2/3 of the genome before synthesis of the other strand is initiated from the other origin. The partially unpaired heavy strand is therefore single stranded during this period, and might be available to mis-pair with the newly synthesized DNA, resulting in the deletion of intervening sequences. This explanation (replication slippage) is weakened by the fact that during this period the complementary strand is not single stranded. Alternative mechanisms of replication such as discontinuous bidirectional DNA synthesis are not well documented features of mtDNA (section 3.1.3). However, Schon et al. (1989a) suggest that "bubbles" may open up where ssDNA becomes available for slipped mispairing may occur, and that sequence features such as homopolymeric tracts may "bend" DNA and exacerbate this. Defects in the normal enzymes and other molecules involved in replication (which are of course, encoded in the nucleus) may explain the autosomal dominant

Errata

(Pages 4, 93, 98, 103, 108). The point mutation described in patient 21 with Leber's Hereditary Optic Neuropathy has not been confirmed. Subsequent work demonstrated this was sequencing artifact.

Fig 10.1

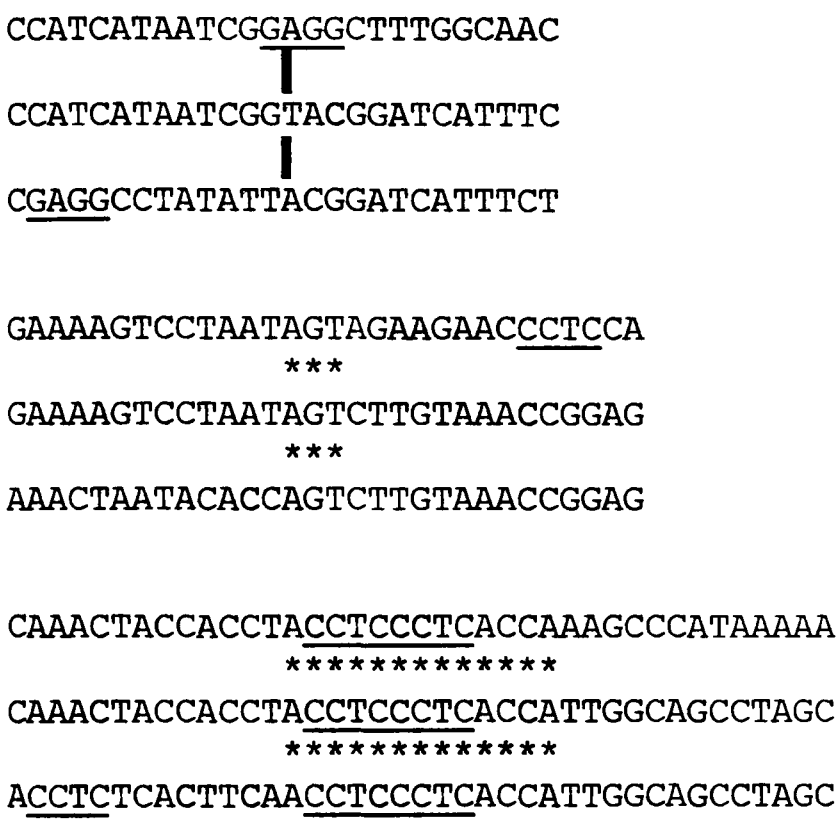


Fig 10.1

Comparison of junction sequences in duplications and the common deletion
Top line: In patient 1, Cytochrome oxidase subunit 1 sequence at bp 6130 adjoins bp 15056 (apocytochrome B)
Middle line: In patient 2, at bp 7354 Cytochrome oxidase subunit 1 sequence adjoins bp 15913
Bottom line: common deletion. There is an exact homology betweem bp 8,470-8,482 (top strand) and 13,447-13,459 (bottom strand).
Key: ▌ represents breakpoint (patient 1)
* homologous region (patient 2 and common deletion)
Underlines indicate Picolli sequence (CCTC or GAGG) sequences

inheritance pattern of the variable deletions described by Zeviani et al. (1989a) (section 3.4.1). Similarly, nuclear genes may be involved in the origins of the duplications described in lizards (Moritz and Brown 1987).

There is no clear molecular mechanism for the rearrangements where there is only a few base pairs or no homology. Inspection of these nucleotide sequences does not reveal obvious features present in all deletions. However, the sequence CCTC or GAGG described by Picolli et al. (1984) which has been implicated in rearrangements of nuclear DNA by Hurst and Porteous (personal communication) frequently occurs at or near either end of the deletions. It is possible that these could represent some kind of weak recognition sequences for DNA binding proteins which might mediate recombination. While no candidate protein has yet been clearly identified, there is at least one endonuclease of unknown function in mammalian mitochondria (Low et al. 1988). Schon et al. (1989b) have also found a possible homology to the topoisomerase II recognition sequence in drosophila in some of these sequences.

In attempting to define characteristics common to major rearrangements of mtDNA, it has been noted that the region of mtDNA where the majority of deletions are found is similar to the non-duplicated region in mtDNA duplications. As dimers (a double-length mitochondrial genome, formed from two copies joined end to end) are present in low levels in normal individuals (Clayton and Vinograd 1967), one could speculate that duplications arose from deletions of normal dimers, and that the mechanism underlying these mutations was very similar. Insights may be obtained from better characterised systems of re-arrangements of mtDNA in other species. For example, mtDNA mutations are able to cause cytoplasmic male sterility in plants (section 3.2.3). Duplications appear to arise via a number of recombination events: inferred "subgenomic intermediates", or deleted molecules appear to recombine to produce duplicated genomes (Small et al. 1989). Thus both deleted and duplicated molecules would be present in the same organism, at least in theory. Support for these ideas comes from the data on Pearson's syndrome. The deletion-insertion described by Rotig et al. (1989b) would be explained by this mechanism. Secondly, they have described a patient in whom they found dimers of deleted molecules, which could be an intermediate form. In addition, we have preliminary evidence that tissue from

these patients may contain high molecular weight forms which include rearranged molecules (section 7.6).

The majority of cases of MM with mtDNA deletions appear to be sporadic. Because the proportion of deleted mtDNA is much higher in muscle than in blood in these patients, this led to the suggestion that these deletions are somatic and not present in the germline. Further work using more sensitive techniques such as PCR showed that although the proportion was highest in muscle, patients with KSS had deleted genomes present in most or all tissues sampled (in contrast to patients with CPEO where this was not easily demonstrated and the deletions appear to be more closely associated with muscle). Many workers have therefore suggested that the deletions must occur during embryogenesis if they are to have a wide tissue distribution, and maybe later in the more limited distribution found in CPEO. A family with KSS and mtDNA deletions has recently been described (chapter 8 and Holt 1989), in whom an identical deletion is detectable in proband, sib and mother, suggesting that the deletion is present in the germline in this family. However, it remains possible that this arose de novo in each of the three individuals, as presumably occurs in the autosomal dominantly inherited mtDNA deletions of Zeviani et al. (1989a).

Given that the severity of symptoms has some relationship with the "dose" of abnormal genomes within a tissue (Wallace et al 1988), what determines their tissue distribution, and in particular the greater proportion of deleted genomes in muscle than blood? In sporadic cases, the explanation could be the occurrence of somatic mutations in muscle: the deletion occurs at a developmental stage where there has already been some partitioning, so that deleted genomes do not spread to all other tissues. However, the germline mutation described in section 8.6.4 requires further explanation. Is it just pure chance that both the phenotype and the tissue distribution of deleted mtDNAs in this patient is markedly similar to the phenotype and distribution in other deletion patients? As discussed in section 3.1.4, in normal individuals a "bottle-neck" appears to operate: only one or a few out of the many thousands of mitochondria present in the ovum appear to divide to populate the organism. However, the widest variation in the ratio of two different mtDNA types between tissues of an apparently "neutral" mutation in a heteroplasmic cow was 1.5 fold (Hauswirth and Laipis 1985). In the case of patient 1 (chapter 8) the difference is probably

in excess of 10 fold. It is therefore most unlikely that the tissue distribution in this patient is merely the result of the "bottleneck" followed by random segregation as described above: other factors may also be operating.

The reason that these rearranged genomes do not appear to conform to this rule, may be that they confer a number of properties on the individual mitochondrion, cell, tissue or organism. For example, the rate of propagation of the deleted mtDNAs could be different from the normal: the replication rate of deleted mtDNAs may be faster, consistent with the increasing proportion of these "selfish" mtDNAs over time (Oldfors et al 1989), as may occur in yeast (De Zamaroczy et al. 1981, see section 3.2.3), the rate of organelle and cell division could be adversely affected by its deleted population as has been observed in cell culture (Moraes et al. 1989a). Selection may operate at the level of the tissue or the organism, if other tissue distributions are lethal: Pearson's syndrome appears to be one such non-lethal distribution (Rotig et al. 1988, section 3.4.2). Alternatively, a mechanism by which an apparently "selfish" mtDNA might be selected to divide actively at the expense of the selfless cell (whose restricted growth may protect the organism), could be a nuclear gene product such as has been implicated by Fangman et al (1990) in yeast (see section 3.2.3).

What then is the explanation for the lack of symptoms in the sibling of patient 1? Why does she appear to have a lower "dose" of deleted mtDNAs when they were also present from conception? It is possible that there is germline mosaicism: different oocytes may contain varying proportions of deleted mtDNAs, and by chance she inherited a lower "dose" or effective "dose" in the developing embryo. Even if some of these speculations about non-random segregation of deleted mtDNAs are correct, chance must also be operating.

Thus, there may be factors affecting tissue distribution which act at the level of the individual mtDNA, the organelle, the cell, the tissue and the whole organism. These are likely to be of central importance in pathogenesis of the disease.

10.6 Applications to clinical genetics

Although in the past attempts have been made to infer mutations of mtDNA from work on mitochondrial proteins, clearly mtDNA analysis is now the investigation of choice. It is hoped that diagnosis based on DNA analysis will

eventually enable a new and simpler classification of this group of diseases. The application of these techniques firstly to diagnosis and carrier detection and then to antenatal diagnosis will be discussed.

Although duplications of mtDNA with KSS and rearrangements in Pearson's syndrome may be detected in blood using Southern hybridisation, most deletions in KSS require muscle biopsy. In order to develop a method for non-invasive diagnosis and carrier detection, PCR was used to detect deletion sequences in blood from patients with mitochondrial myopathy, using primers designed to detect the "common deletion" (chapter 8). In 8 patients and 2 of their relatives, PCR products were obtained consistent with deletions of mitochondrial DNA. The presence or absence of a deletion was correctly predicted in 10 out of 11 patients from whom information was available from muscle DNA. No false positives were obtained in 43 controls, and all the PCR products suggestive of the common deletion were sequenced to confirm their inferred origin. In one family, the deletion sequence was detected in two asymptomatic individuals, who must therefore be at risk for transmitting the disorder. We believe that this may become very useful in clinical practice.

As it stands the protocol used appears to be reliable. Clearly, sequencing of PCR products is not a possibility for a routine laboratory, but the products could be roughly identified by restriction enzyme analysis or with reasonable certainty by oligonucleotide probing. Modifications using alternative primers would require extensive controls, because of the problems of false priming. Replacing the use of nested primers with a single pair placed nearer to the location of the common deletion might prevent detection of the slightly larger deletions detected in patients 4 and 8, and contamination would be more likely to confuse results.

Despite the advances in understanding of mitochondrial mutations in human disease, antenatal diagnosis has not yet been feasible in the majority of mutations, for two reasons. In the first place, the majority of cases in whom mitochondrial rearrangements have been described are sporadic, and hence transmission of the phenotype uncommon. Secondly, there is a wide variation in the degree of heteroplasmy in different tissues of the patients with the rearrangements discussed above. As the mechanisms whereby this variation arises are not well understood, it is possible that a biopsy of embryo or chorionic

villus might not be representative of the remainder of the foetus: the proportion of abnormal genomes might thus be under- or over-estimated. Until more is known about the segregation of mtDNA during embryogenesis, it will be hard to base genetic advice on biopsy information. However, the variation in tissue distribution in patients with point mutations may not be so wide. There is thus a single unpublished report of antenatal diagnosis and abortion of an affected foetus in a family with a mtDNA point mutation and myopathy (Harding AE, personal communication).

10.7 Future prospects

Much more work is required to answer many of these points. Although it is now extremely likely that rearrangements of mtDNA cause disease in man, strictly speaking no more than a close association between the two has been described. Fulfilment of the final Koch postulate would require the production of some aspect of the phenotype, such as the respiratory defect, by introduction of rearranged mtDNA into a recipient cell line or organism. King and Attardi (1989) have developed a cell line free of mtDNA, into which they can introduce mitochondria either by cell fusion or by injection of single mitochondria. This technique may allow the ultimate test. Alternatively, it is possible to transform yeast by bombardment with microprojectiles (Johnson et al. 1988), and this might be applicable to human cell lines. Another approach would be to introduce the fusion genes resulting from mtDNA re-arrangements (such as those described in patients 1 and 2 of chapter 7) into cell lines (preferably stem cell lines) so that they were transcribed and translated (necessitating changing from mitochondrial to nuclear genetic code) and imported into mitochondria (necessitating the addition of an import sequence to the gene), so investigate phenotypic effects.

As far as the experimental work in this thesis is concerned, there are several gaps which will be filled in the near future. The biochemical effects of the duplications described in chapter 7 are currently under investigation: Western blotting of purified mitochondria from various post-mortem samples from patient 1 is in progress. If there is no evidence of the predicted abnormal cytochrome oxidase subunit 1 using the available antibodies, polyclonal antibodies will be raised against the 20 amino acid residue sequence resulting

from the frame shift. Clonal fibroblast lines are being raised, and if a high proportion of duplicated genomes is obtained, it may be possible to investigated their transcriptional and translational activity and function in more detail. Thirdly, quantitation of duplicated mtDNAs and high molecular forms in the post mortem tissue from patient 1 and its relation to histology and symptoms may widen our understanding of the factors involved in segregation of mtDNA. Fourthly the high molecular weight forms should be mapped in more detail and their presence sought in more patients.

The applicability of the PCR protocol in chapter 8 to clinical practice should be widened by using other combinations of primers to detect a wider range of deletions in blood.

Identification of new mutations in patients with Leber's hereditary optic neuropathy such as that described in patient 21 chapter 9 may help confirm the importance of the Wallace mutation in the pathophysiology.

10.8 Conclusion

In conclusion, investigation of the functional activity of these abnormal mitochondrial genomes will help to clarify their relationship to the clinical phenotype in these diseases.

Practical benefits will include new approaches to diagnosis and classification, improvements in genetic counselling and assessment of prognosis, and may ultimately include new approaches to treatment.

Investigation of other conditions mentioned in section 3.4 such as congenital myotonic dystrophy may demonstrate a larger role for mtDNA in human disease than so far described.

In the long term it can be anticipated that knowledge of the pathology of this genome will illuminate aspects of its normal function in mitochondrial biogenesis.

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Appendix 1

Clinical details and reference numbers of patients appearing in both chapters 6 and 8

Patient no. Ch6	Ch8	Sex	FH	Biochemical defect	Deletion PCR on blood	Muscle blot	Category
1	3	M	-	I	No	NA	CPEO
2	5	M	-	NA	No	No	CPEO
3	1	M	*	-	Yes (cd)	Yes	KSS
4	4	M	-	I & IV	Yes	Yes	CPEO
5	6	F	-	-	Yes (cd)	Yes	CPEO
6	13	F	+	I	No	No	II
7	19	F	-	NA	No	NA	MELAS
8	20	F	-	I	No	No	MELAS
9	23	M	-	V & CN	No	NA	MERRF
10	24	F	-	III & IV	No	No	MERRF

Appendixll: Human gene nomenclature for the mitochondrial genome

Gene symbol	Marker name	Strand H, heavy L, light	Nucleotide location
MTATP6	ATPase subunit 6	H	8,527-9,207
MTATP8	ATPase subunit 8	H	8,366-8,572
MTCOX1	cytochrome c oxidase subunit I	H	5,904-7,444
MTCOX2	cytochrome c oxidase subunit II	H	7,586-8,262
MTCOX3	cytochrome c oxidase subunit III	H	8,207-9,990
MTCYB	cytochrome b	H	14,747-15,887
MTHSP	H-strand promoter	H	545-567
MTLSP	L-strand promoter	L	392-435
MTND1	NADH dehydrogenase subunit 1	H	3,307-4,262
MTND2	NADH dehydrogenase subunit 2	H	4,470-5,511
MTND3	NADH dehydrogenase subunit 3	H	10,059-10,404
MTND4	NADH dehydrogenase subunit 4	H	10,760-12,137
MTND4L	NADH dehydrogenase subunit 4L	H	10,470-10,766
MTND5	NADH dehydrogenase subunit 5	H	12,337-14,148
MTND6	NADH dehydrogenase subunit 6	L	14,149-14,673
MTOHR	Origin of H-strand replication	H	about 200
MTOLR	Origin of L-strand replication	L	5,729-5,805
MTRNR1	12S rRNA	H	648-1,601
MTRNR2	16S rRNA	H	1,671-3,229
MTTA	tRNA, alanine	L	5,587-5655
MTTC	tRNA, cysteine	L	5,761-5,826
MTTD	tRNA, aspartic acid	H	7,518-7,585
MTTE	tRNA, glutamic acid	L	14,674-14,742
MTTF	tRNA, phenylalanine	H	577-647
MTTG	tRNA, glycine	H	9,991-10,058
MTTH	tRNA, histidine	H	12,138-12,206
MTTI	tRNA, isoleucine	H	4,263-4,331
MTTK	tRNA, lysine	H	8,295-8,364
MTTL1	tRNA, leucine-1	H	3,230-3,304
MTTL2	tRNA, leucine-2	H	12,266-12,336
MTTM	tRNA, methionine	H	4,402-4,469
MTTN	tRNA, asparginine	L	5,657-5,729
MTTP	tRNA, proline	L	15,955-16,023
MTTQ	tRNA, glutamine	L	4,329-4,400
MTTR	tRNA, arginine	H	10,405-10,469
MTTS1	tRNA, serine-1	L	7,445-7,516
MTTS2	tRNA, serine-2	H	12,207-12,265
MTTT	tRNA, threonine	H	15,888-15,953
MTTV	tRNA, valine	H	1,602-1,670
MTTW	tRNA, tryptophan	H	5,512-5,576
MTTY	tRNA, tyrosine	L	5,826-8,891

Publications appended

Poulton J, Turnbull DM, Mehta AB, Wilson J, Gardiner RM, 1988, Restriction enzyme analysis of the mitochondrial genome in mitochondrial myopathy. J Med Genet 25,
600-605.

Poulton J, Deadman ME, Gardiner RM, 1989, Duplications of mitochondrial DNA in mitochondrial myopathy.
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Tandem direct duplications of mitochondrial DNA in mitochondrial myopathy: analysis of nucleotide sequence and tissue distribution

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ABSTRACT

Two patients with direct tandem duplications of mitochondrial DNA (mtDNA) and mitochondrial myopathy are described. The breakpoint regions between duplicated segments were amplified using the polymerase chain reaction (PCR), cloned and sequenced. The distribution of normal and abnormal genomes in different tissues was investigated using Southern hybridisation, and in different cells within the same tissue using PCR. In each case the gene for cytochrome oxidase subunit I (MTCOX1) was interrupted, creating reading frames which if transcribed and translated would result in truncated versions of this peptide. Heteroplasmy and mosaicism for the abnormal mtDNA population was apparent.

INTRODUCTION

In the past year, mutations of mitochondrial DNA (mtDNA) have been described in association with human disease: deletions in mitochondrial myopathy (MM)[1 2 3] and Pearson's syndrome[4], and a point mutation in Leber's hereditary optic neuropathy (LHON)[5].

We have recently described two patients with Kearns–Sayre syndrome (KSS) and diabetes mellitus, in whom tandem direct duplications of mtDNA were demonstrated using Southern hybridisation[6]. Unlike the cases with deletions, in these patients abnormal genomes were easily detectable in blood as well as in muscle, and they were demonstrated in every tissue examined using the polymerase chain reaction (PCR)[7]. PCR was also used to amplify segments of DNA spanning the abnormal junction between duplicated portions in order to map the location of the deletions more precisely. We now present sequence data from these products, and further quantitation of the tissue and cellular distribution of the abnormal genomes.

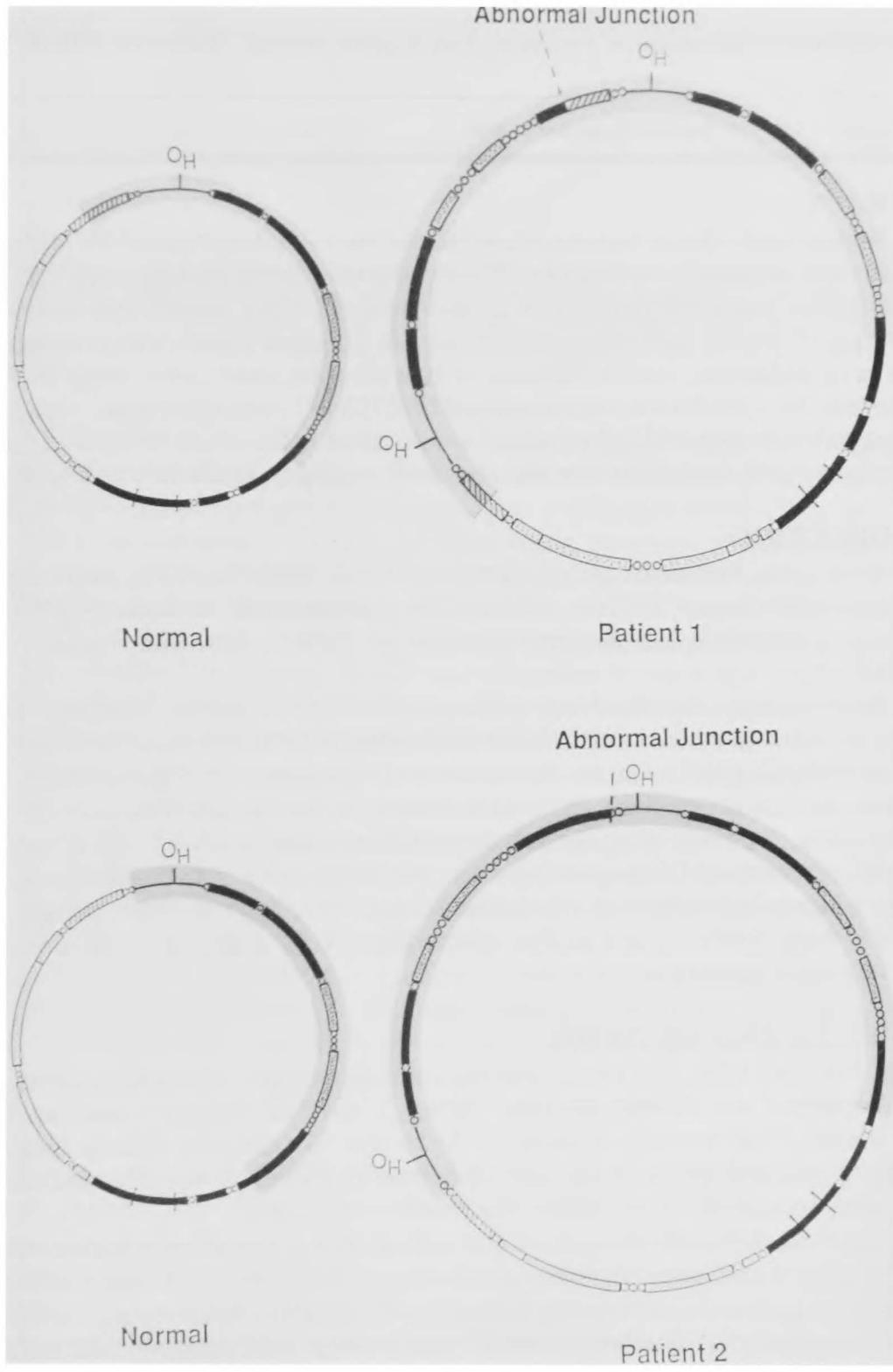
MATERIALS AND METHODS

Patients: Both had KSS, with ptosis, external ophthalmoplegia, retinopathy, ataxia, proximal muscle weakness and diabetes mellitus. Patient 1 was deaf and had a cardiac conduction defect. Patient 2 was mentally retarded and had stroke-like episodes. Muscle biopsy showed classical ragged red fibres in the case of patient 1. Patient 2 was biopsied early in her illness, and no specific histological abnormality was noted.

Samples and Southern hybridisation: Total cellular DNA was extracted from whole blood and from EBV transformed lymphocyte lines[8], and from muscle, urinary epithelium and fibroblasts in patient 1[9]. DNA was digested with BamHI (Amersham), electrophoresed in 0.8% agarose gels, Southern blotted[10] and probed with purified total mtDNA. The

relative proportions of normal and abnormal genomes were then measured densitometrically using a scanning laser densitometer. Clonality was investigated using DNA extracted from single hairs[11] or granulocyte clones[12] from patient 1. PCR was carried out using primers across the abnormal junction (D5 or D3 and CO1/1 see fig1), and with control primers CO1/1 and CO1/4 in a separate reaction to confirm the presence of normal MtDNA available for amplification.

a



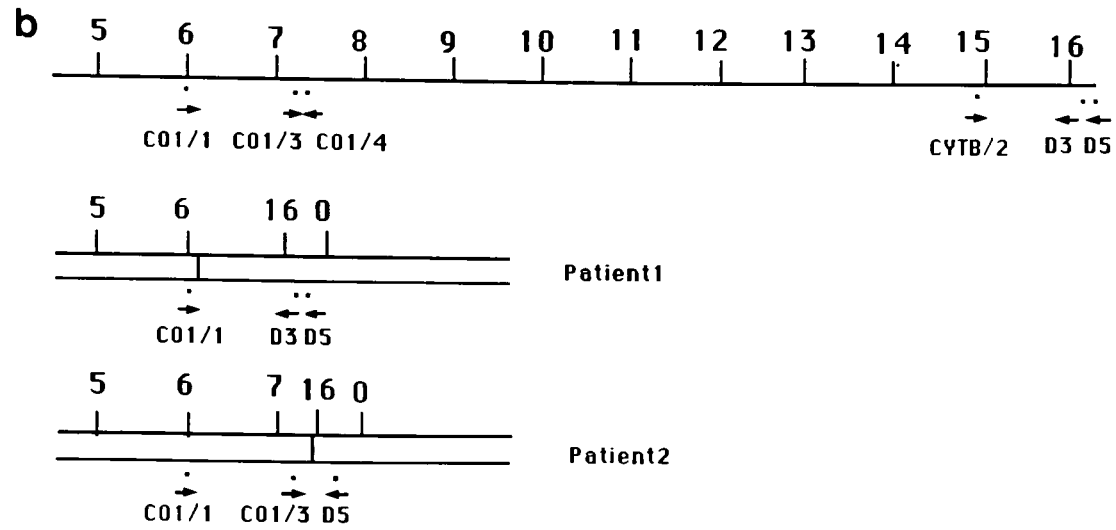


Figure 1. a) Rearrangements of mtDNA in patients 1 and two. In each case the shaded region represents the duplicated portion. O_H: Origin of replication of the heavy strand. For genes involved in the duplicated region see text. b) Location of oligonucleotide primers in relation to the breakpoints in patients 1 and 2. Primers CO1/1, CO1/3, ND5/2 are 20mers corresponding to base numbers 6004 to 6023, 7193 to 7212, and 13,707 to 13726 respectively. Primers Cytb/3, D3 and D5 are complimentary to base numbers 15,132 to 15151, 16,259 to 16,278, and 16,401 to 16,420.

Polymerase Chain Reaction: Oligonucleotide primers 20 bp in length were designed to be complementary to mtDNA sequences[13] either side of the abnormal junction (see fig 1). Thermostable Taq polymerase (Cetus corporation) was used with buffers and dNTP concentrations as recommended ($MgCl_2$ 1.5mM), and a computer controlled alternating temperature water bath (courtesy of Dr Tom Kocher). Denaturation was at 93°C for 1 minute, annealing at 37°C for 1 minute and extension at 73°C for 2.5 minutes for 30 cycles. In the case of patient 1, PCR was carried out using primers CO1/1 and D3 to obtain a 1.3kb product for cloning. In the case of patient 2, the 1.8kb product of PCR using CO1/1 and D5 was used. Contamination was excluded by simultaneous PCR on controls, unaffected relatives and 21 other patients.

Cloning: PCR products were separated from unincorporated dNTPs using a Centricon separator. Any ragged ends of the PCR products were filled in with the Klenow fragment of DNA polymerase 1, cloned into a modified bacteriophage M13 mp8[14] using standard conditions for cDNA cloning. The ligated product was then used to transform *E.coli* JM101 using the Hanahan[15] protocol. White plaques were screened by crude extraction of ssDNA using EndoRstop buffer and electrophoresis.

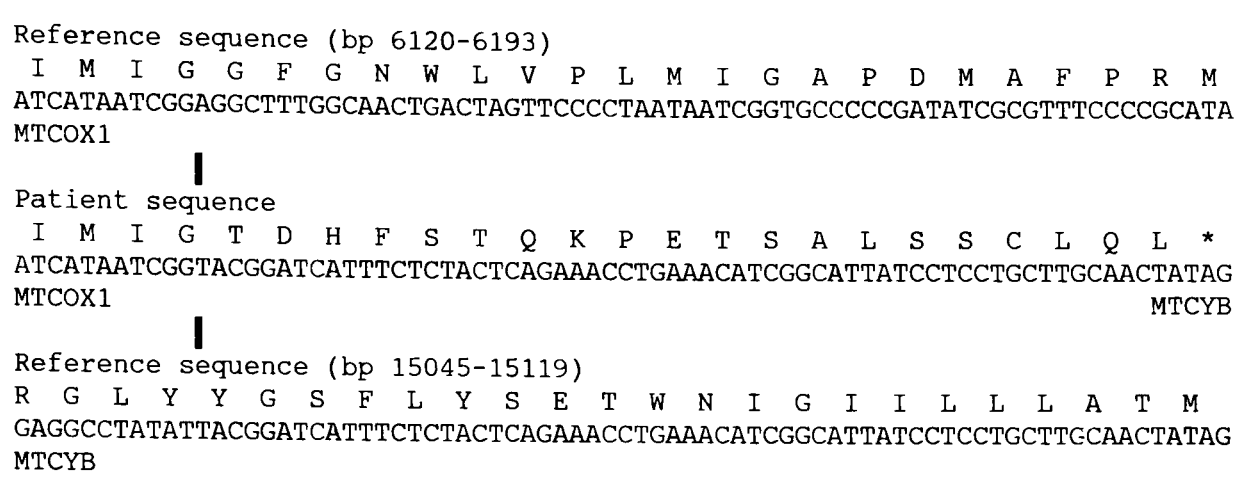
Sequencing: DNA was sequenced by the dideoxy method using Klenow fragment, using the universal m13 primer in patient 1, and sequencing primer CO1/3 (see fig) in patient 2. Sequence was analysed using the Staden package and the EMBL database.

RESULTS

Sequencing

A single clone was obtained for each patient. Sequencing confirmed the preliminary mapping data for both patients (fig 2). In both cases the gene for cytochrome oxidase subunit I is interrupted: by apocytochrome b in patient 1, and by tRNAthr in patient 2. The mtDNA of both patients is therefore duplicated for about half of the total length of DNA, and carries two complete copies of the genes for NADH dehydrogenase subunits 1 and 2, for both rRNAs, for several tRNAs, the D-loop and both origins of replication. In the case of patient

a) Patient 1



b) Patient 2

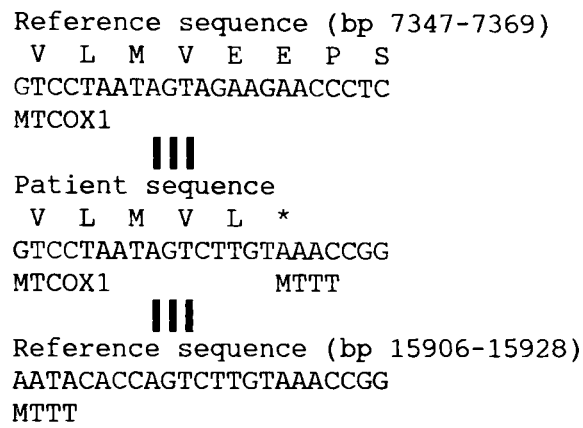


Figure 2. Sequence data: Reference sequence of parent strands above and below the sequence obtained and inferred amino acid sequence of any translated peptide in a) patient 1 (sequence for bp 6130 adjoins bp 15056) and b) patient 2 (sequence for bp 7354 adjoins bp 15913). Key: ▌ represents breakpoint (patient 1) or homologous region (patient 2), MTCOX1: Cytochrome oxidase subunit1, MTCYB: apocytochrome B, MTTT: $tRNA^{thr}$

1, the reading frame for cytochrome oxidase subunit 1 (MTCOX1) was interrupted at base no 6130 with base no 15056 of apocytochrome b (MTCYB) sequence out of frame without any intervening sequence (fig 2a). If transcribed and translated this would result in a truncated peptide consisting of the first 76 residues of MTCOX1 sequence followed by 20 abnormal residues unrelated to the normal MTCOX1 or MTCYB amino acid sequence before the first stop codon is generated. In patient 2, MTCOX1 sequence is interrupted after base no 7354 with base no 15913 of the tRNAthr sequence without any intervening sequence. There was a homologous portion 3 base pairs in length in both parent strands. If transcribed and translated this would result in a peptide corresponding to the first 497 amino acid residues of MTCOX1 followed by one abnormal residue before the first stop codon is generated.

Table 1: Proportion of duplicated mtDNAs in various tissues.

Tissue	Mitochondrial genome		Proportion of clones in which duplication detectable
	% duplicated	%normal	
Patient 1 Blood	32%	68%	
Cloned granulocytes	NA	NA	3/5 (60%)
Muscle	20%	80%	
Hair—Pooled	<5%	>90%	
—Individual	NA	NA	5/16 (31%)
Urinary epithelial cells	<5%	>95%	
Patient 2 Blood	43%	57%	

In order to investigate the possibility that reciprocal deleted genomes might be present corresponding to the DNA portion effectively deleted between the duplicated segments PCR was also carried out using primers CO1/4 and Cytb2. If these genomes existed they should give rise to products 1.4 and 1.1 kbp in length. No product was obtained although each primer was able to generate product when used in other appropriate combinations.

Tissue distribution

Densitometry revealed that normal and abnormal mtDNAs comprised approximately 32 and 43% of the total mtDNAs in blood in both cases 1 and 2 respectively (table 1). Patient 1 had 20−25% abnormal genomes in muscle. No muscle tissue was available for patient 2. Southern hybridisation failed to detect abnormal genomes in hair, fibroblasts, or bladder epithelial cells, but these were detectable using PCR (see table), suggesting that they were present at low levels. The relative quantities of PCR product from individual hairs and granulocyte clones suggested that the proportions of normal and abnormal mtDNAs varied in different cells within the same tissue.

DISCUSSION

The sequence data on these two patients confirms the mapping data, and taken together these demonstrate tandem direct repeats. A single clone was sequenced in both cases, and it is possible that additional populations of mtDNA exist in these patients[16]. As there was no evidence for this on either PCR or Southern hybridisation, any such populations must have similar rearrangements or be present in low levels.

As none of the PCR products from the small tissue samples was sequenced, it is not absolutely certain that they represent the identical duplication. However, the products were all the same size as those from blood which were sequenced.

Of the tissues investigated, duplicated mtDNAs were only detectable using Southern hybridisation in blood and muscle. PCR revealed that they were present in every other tissue investigated, and although not strictly quantitative it is reasonable to suppose that the variation in quantity of product between different hairs and granulocyte clones may represent differences in the proportion of abnormal genomes in different clonal lines. Surprisingly, the proportion of abnormal genomes was higher in blood than muscle in patient1. However, it must be remembered that the muscle sample was obtained 9 years before the blood sample during which time her muscle power deteriorated. It may be that

the duplicated genomes have a replicative advantage over the normal. Moreover, as only one muscle was biopsied it might not be representative of the overall distribution.

What is the relationship of these mutations to the clinical phenotype? Their absence from normal family members and from large population studies argues that they are causal although the mechanism is uncertain. Mitochondrial function could be impaired by an abnormal product of the rearranged genes, or by a relative excess of normal products. In both of our patients, one of the two copies of mtCOX1 is interrupted, and if transcribed and translated these fusion genes would give rise to truncated CO1 peptides 96 and 485 amino acid residues in length instead of the normal 513 residues. These might compete with the normal subunits prevent the normal assembly of cytochrome oxidase and hence lower its activity. There is a precedent for this in cytoplasmic male sterility in petunia[17]. Alternatively, the duplicated regions might cause harm by resulting in an excess of the products they encode (for instance protein subunits of complex 1, or transcripts such as rRNAs and tRNAs) which might interfere with correct RNA processing, or unbalance the kinetics of multimer assembly. However, duplications in a similar region in lizards[18] appear to be phenotypically silent, making the former slightly more likely.

The mechanism by which the duplications have arisen is not clear. Tandem direct duplications are equivalent to deleted dimers, in this case deleted by 8−9kb. Dimers may rarely occur during normal replication[19], and an 8−9kb deletion in one of these could have given rise to these duplications although there is very little homology at the breakpoint in the parent strands[20]. Alternatively, duplication could result from recombination with a transducing deleted mtDNA[21]. However, mtDNAs with the reciprocal deletion which would be predicted in either case were not detectable using 30 cycles of PCR, and if present must be a low proportion. As such mtDNAs would not contain any origins of replication and might not replicate, their apparent absence does not exclude either mechanism. It may be noted that the sequence 'GAGG' or 'CCTC' which has recently been implicated in rearrangements of nuclear DNA by Picolli et al[22] is present at or near the breakpoint in two parent strands in patient 1, and one in patient 2.

Further work on rearrangements of mtDNA in health and disease will provide new insights into mitochondrial function.

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Restriction enzyme analysis of the mitochondrial genome in mitochondrial myopathy

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Restriction enzyme analysis of the mitochondrial genome in mitochondrial myopathy

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SUMMARY The mitochondrial myopathies are a heterogeneous group of disorders some of which may be caused by mutations in the mitochondrial genome. Mitochondrial DNA from 10 patients with mitochondrial myopathy and their mothers was analysed using five restriction enzymes and 11 mitochondrial probes in bacteriophage M13. No abnormalities were found in seven out of the 10 patients. Polymorphisms which have not previously been reported were detected in three patients and two of their mothers. These results exclude the presence of deletions or insertions of greater than 60 bp in the region of the mitochondrial genome examined. Any causative mitochondrial DNA mutations in these disorders are therefore likely to be point mutations or small structural rearrangements.

The mitochondrial myopathies comprise a clinically heterogeneous group of disorders characterised by the accumulation of structurally abnormal mitochondria in skeletal muscle.[1–3]

Biochemical studies on mitochondria isolated from skeletal muscle have identified defects at a number of sites including complex I (NADH-ubiquinone reductase), complex III (ubiquinol-cytochrome C oxido-reductase), complex IV (cytochrome oxidase), and ATPase.[1 3]

The genetic basis of these disorders is uncertain. Of particular interest is the occurrence of a high incidence of maternal transmission in some families.[3–5] Indeed, there is one extensive pedigree with nine affected subjects and exclusively maternal transmission not explicable by autosomal or X linked recessive inheritance, where the authors reported the probability of autosomal dominant inheritance to be as low as 0·0005.[4] As mitochondrial DNA is maternally inherited,[6] this observation led to the suggestion that the pattern of inheritance reflected the transmission of an abnormality within mitochondrial DNA.[4] While the majority of components of the respiratory chain are encoded in the nucleus, the mitochondrial genome is known to code for apocytochrome B, and some subunits of cytochrome oxidase, ATPase, and complex I.[7 8] Functional abnormalities of these components have been identified in some patients with mitochondrial myopathy. It is possible, therefore, that in some of these patients the causative mutation lies within the mitochondrial rather than the nuclear genome.

Approaches to investigating this problem include analysis of the mitochondrial protein products, which poses considerable technical problems, or of the mitochondrial DNA. The elucidation of the complete sequence of human mitochondrial DNA by Anderson *et al*[7] makes DNA analysis an appropriate alternative. Restriction mapping may be used to identify major alterations and to survey in detail that small segment of the base sequence comprising the enzyme recognition sites. However, DNA sequencing is necessary to exclude smaller changes, such as point mutations, throughout the genome.

In this study restriction mapping has been used as the necessary preliminary investigation to exclude major DNA alterations in 10 patients with mitochondrial myopathy and their families. Eleven probes, including those corresponding to regions coding for relevant protein subunits, were used in order to maximise unambiguous regional detail (fig 1).

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Patients

Ten patients with mitochondrial myopathy were studied. Details of their clinical features, together with the results of histological and biochemical investigations are shown in the table. Nine of the patients' mothers were alive and available for study. One had died from mitochondrial myopathy, and in one case the mother was affected, as demonstrated by histological criteria, and probably both sibs (fig 2). Six patients have been reported previously, as indicated. Normal subjects were included as controls. Ethical permission was granted by the Central Oxford Research Ethics Committee.

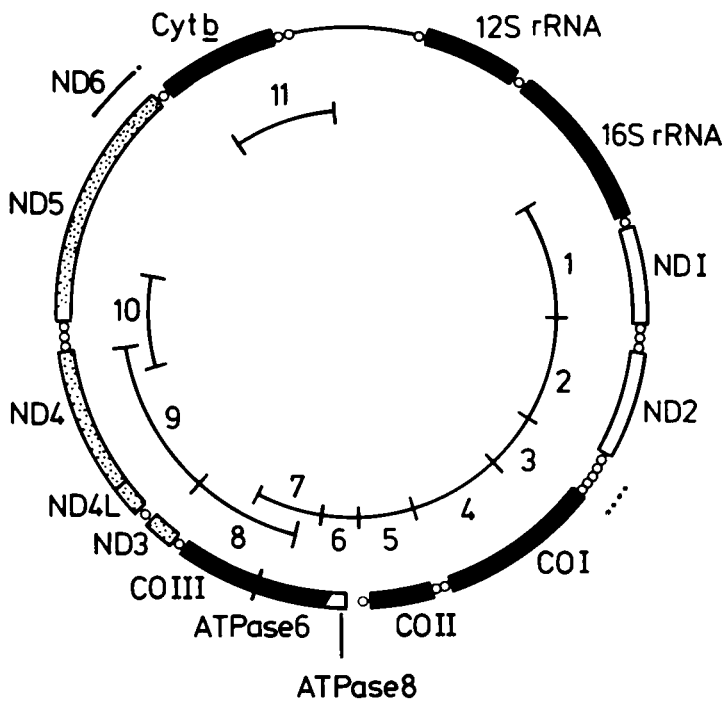

FIG 1 *The human mitochondrial genome showing location of probes used. COI to COIII: subunits 1 to 3 of cytochrome C oxidase. ND1 to ND6, and ND4L. subunits 1 to 6 and 4L of NADH-dehydrogenase. ATPase 6 and 8: subunits 6 and 8 of H^+-ATPase. Cyt b: apocytochrome B. 1–11: regions corresponding to probes 1 to 11 referred to in text. O: transfer RNAs.*

Materials

Restriction enzymes were obtained from Boehringer Mannheim, Amersham International, and Pharmacia, and used according to the manufacturer's instructions. Nylon filters (Hybond N) and [α–^{32}P] dCTP (PB 10205, specific activity 3000 Ci/mmol/)

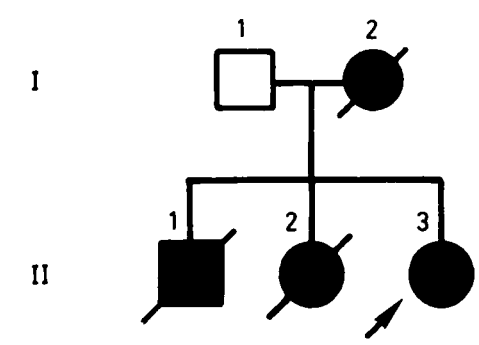

FIG 2 *Pedigree of family 6. Patients I.2, II.1, and III.3 all had a similar disease to the proband, apart from age of onset. They died aged 35, 15, and 10 from respiratory failure. Patient II.3 had progressive pain on exercise and weakness from the age of 17 and is now wheelchair bound.*

TABLE *Patients studied.*

Patient No	*Age at onset (y)*	*Sex*	*Clinical category**	*Family history*	*Investigations*		*References*
					Muscle histology: ragged red fibres	*Site of biochemical defect: complex*	
1	14	M	I	—	+	I	9
2	1	M	I	—	+	—	
3	14	M	III	—	+	NAS	3
4	8	M	I	—	O‡	I & IV	10
5	8	F	I	—	+	NAS	
6	17	F	II	Positive†	+	I	
7	4	F	III (MELAS)	—	+	NAS	
8	9	F	III (MELAS)	—	+	I	11
9	5	M	III (MERRF)	—	+	V (and carnitine deficiency)	3
10	6	F	III (MERRF)	—	+	III & IV	3

*Clinical categories I, II and III.[3]
†Family history (see fig 2).
‡Electron microscopy showed abnormal mitochondrial inclusions. Mitochondrial metabolism was previously studied on mitochondria isolated from skeletal muscl. For methods see references 6, 10, and 11.
NAS, no abnormality seen.
MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes.[2]
MERRF, myoclonic epilepsy and ragged red fibres.[5]

were obtained from Amersham International. Size markers were obtained from Bethesda Research Laboratories and reagents for oligonucleotide labelling were from Pharmacia. A set of 11 probes consisting of double stranded DNA in bacteriophage M13 complementary to specific regions of the human mitochondrial genome were used (fig 1), by courtesy of Professor G Attardi. These probes are complementary to approximately 83% of the protein reading frames and 70% of the total mitochondrial genome.

Methods

Total cellular DNA was prepared from the white cells of EDTA anticoagulated blood by standard methods.[12] DNA was digested with each of the following enzymes: *Alu*I, *Dde*I, *Hae*III, *Hinf*I, and *Taq*I. In selected patients double digests with *Hinf*I and *Hae*III and digestion with *Bst*NI or digestion with *Sty*I were performed. Digested DNA (3 μg) was separated by electrophoresis in 2·2% agarose gels (4 volts/cm) and transferred to nylon filters by Southern blotting.[13]

Probes were labelled to an average specific activity of 1 to 2×10^9 dpm/μg by the hexanucleotide primer method.[14] Hybridisation was carried out for 12 hours at 65°C (10% dextran sulphate, 1% SDS, 5×Denhardt's, 5×SSPE, 100 mg/ml salmon sperm DNA, 125 μg/ml tRNA). Post-hybridisation washes were in 2×SSC, 0·1% SDS and 0·2×SSC, 0·1% SDS at 65°C. Autoradiography (Fuji *x* ray films) with intensifying screens was at −70°C for 12 to 96 hours.

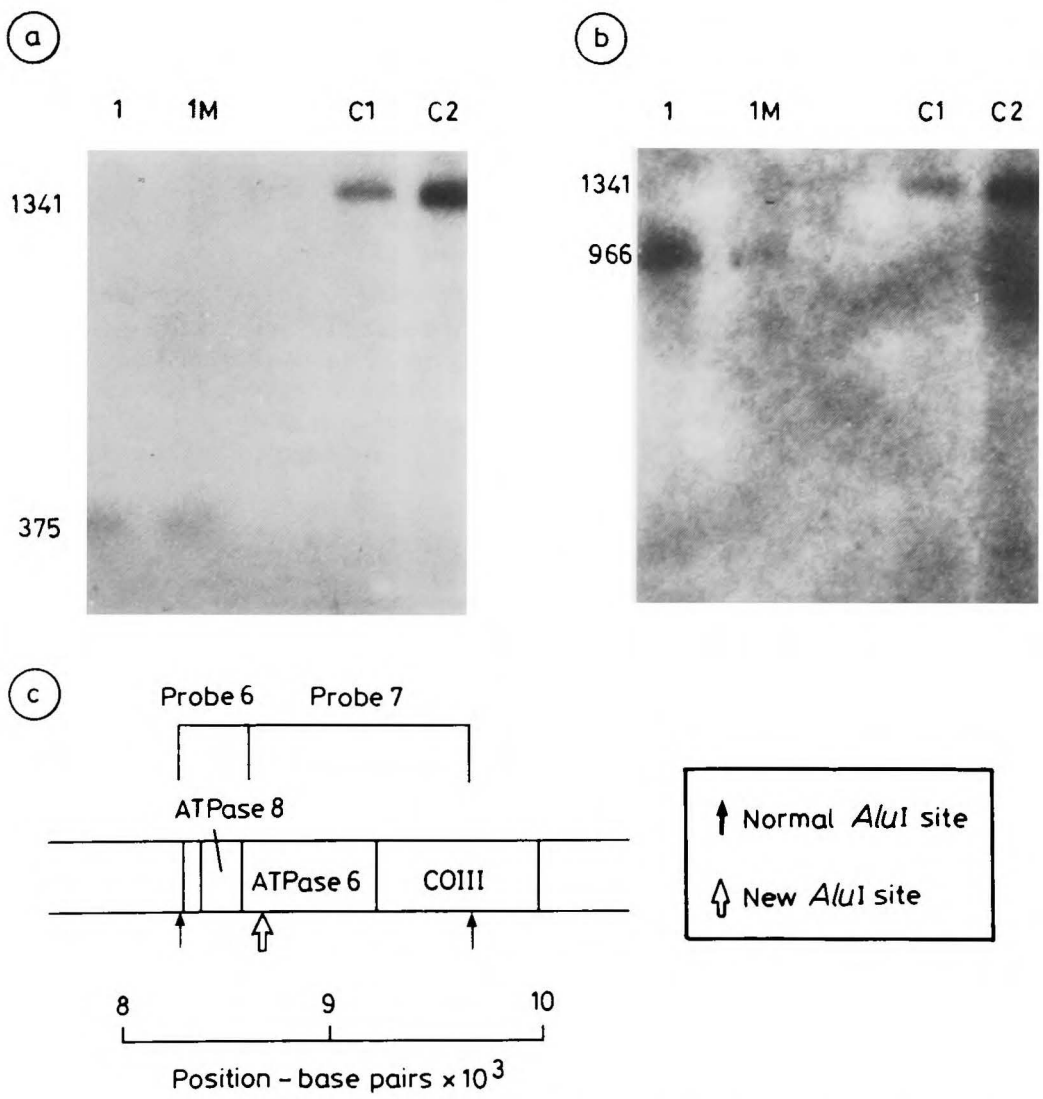

FIG 3 *Site changes in case 1. Autoradiographs of AluI digest of case 1 (1), his mother (1M), and two controls (C1, C2), hybridised with (a) probe 6 showing replacement of the normal 1341 fragment with a 375 fragment and (b) probe 7 showing replacement of the 1341 fragment with a 966 fragment. (c) Restriction map of bases 8305 to 9645 of the mitochondrial genome.*

Results and discussion

The restriction fragment sizes predicted from the human mitochondrial DNA sequence[7] were detected for each combination of restriction enzyme and probe. The smallest fragment size detected was 116 bases. In seven patients the restriction fragments detected did not differ from those predicted from the known sequence and previous population polymorphism data.[7 15] These results exclude the existence of major rearrangement insertions or deletions of greater than 60 base pairs within the region of the mitochondrial genome examined. They also exclude point mutations within the 5% of the portion of the mitochondrial genome examined, which comprised the restriction sites surveyed.

New mutations were identified in three patients and in two of their mothers. In patient 1 and his

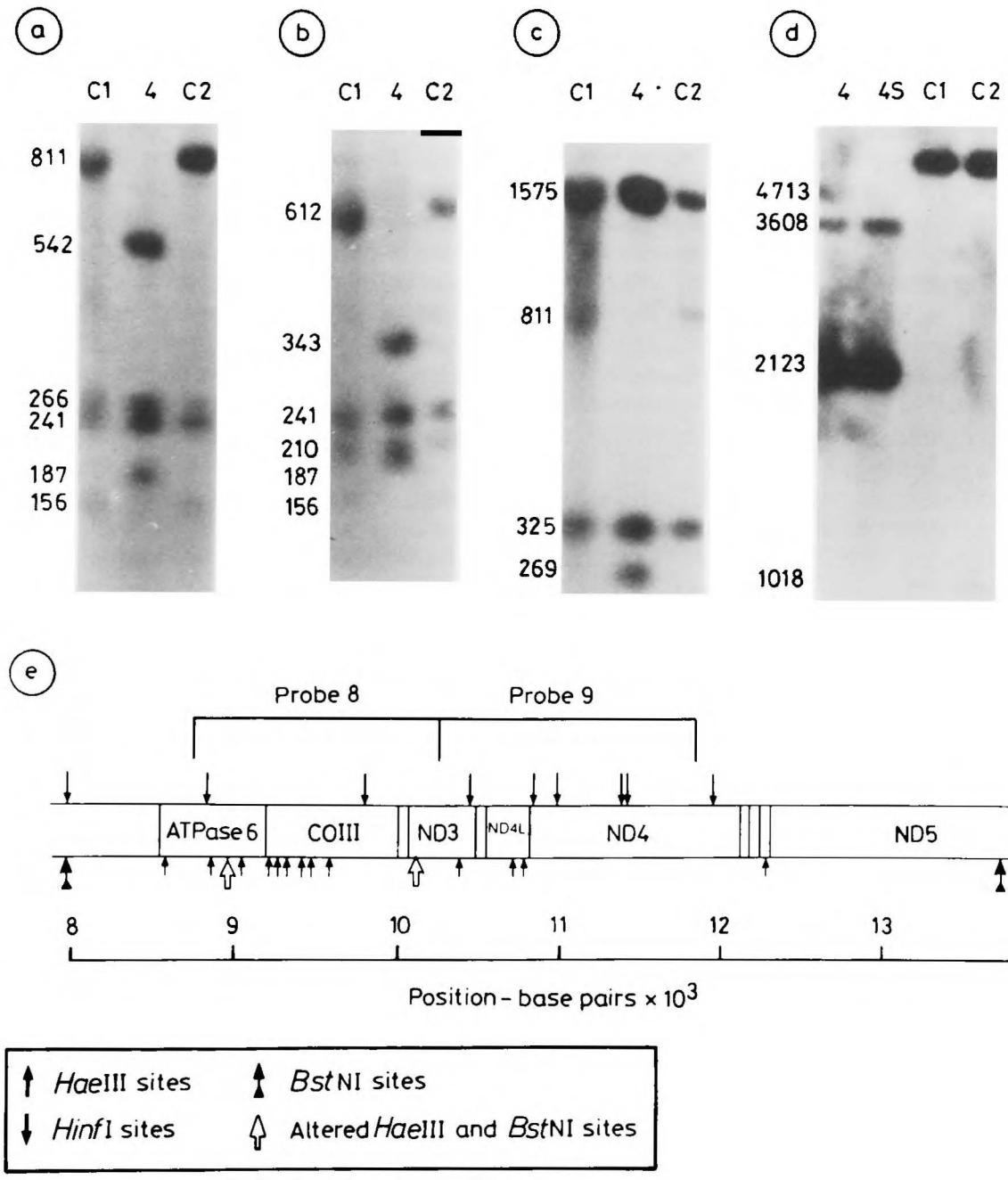

FIG 4 *Site changes in case 4. Autoradiographs of (a) HaeIII digest of case 4 (4) with two controls (C1, C2) hybridised with probe 8 to show replacement of normal 811 fragment by 542 and of normal 187 fragment by 156. (b) Digest with HaeIII and HinfI hybridised with probe 8 to show replacement of normal 612 fragment by a 343 fragment and replacement of 156 fragment by 187 fragment. (c) Digest with HaeIII hybridised with probe 9 to show replacement of 811 fragment by 269 fragment. (d) Autoradiograph of BstNI digest of case 4a (4), his sister (4S), and two controls (C1, C2) hybridised with probe 9 to show replacement of normal 4713 base and 1018 base fragments by 3608 and 2123 fragments. (e) Restriction map of bases 7975 to 13 705 of the human mitochondrial genome.*

mother, digestion with *Alu*I resulted in two new fragments of 375 and 966 bases replacing the normal 1·34 kb fragment. These were shown by probes 6 and 7, respectively (fig 3). Because transitions occur much more frequently than transversions,[16] the most likely position can be inferred by searching for semi sites. This suggests a new *Alu*I site at 8679 base pairs, in the region coding for ATPase subunit 6, caused by an A–G transition which would not result in an amino acid change.

Digestion of DNA from patient 4, his sister, and his mother with *Hae*III showed one new polymorphism and one which has been described previously. The normal 811 base fragment is divided into 542 and 269 base pairs at a new *Hae*III site shown by probes 8 and 9, respectively (fig 4a, c). A double digest with *Hae*III and *Hinf*I suggests that the mutation creating the new *Hae*III site is located at 10 097 base pairs (fig 4b). Secondly, the normal 156 base *Hae*III fragment was replaced by a 187 base fragment (fig 4a), as has previously been described.[15] This is consistent with a *Hae*III site loss at position 8995. In addition, digestion with *Bst*NI and hybridisation with probe 10 (fig 4d) shows replacement of the normal 4713 and 1018 base fragments by two fragments of size 3608 and 2123 bases. This, again, suggests a *Bst*NI site gain around 10 097, and a *Bst*NI site loss around 8995. This indicates that the most likely point mutation at 10 097 is an A–G transition lying within the recognition sites of both *Hae*III and *Bst*NI. This is the third base in a codon in the reading frame of ND3 and would not result in an amino acid change. Similarly, the most likely cause of the *Bst*NI and *Hae*III site losses around 8994 is a G–A transition either at position 8994 or at 8995. The former is the third base in a codon in the reading frame of ATPase 6, and would not result in an amino acid change; the latter would result in replacement of alanine with threonine.

In patient 3, digestion with *Dde*I and hybridisation with probe 11 showed replacement of the 292 band by a 238 band. This site gain could be located at either 15 758 or 15 945, and the latter was excluded by obtaining normal restriction fragments with *Sty*I. Thus, the most likely mutation is a G–A transition at 15 758, which would give rise to replacement of an isoleucine residue with valine.

Neither of these two amino acid changes is likely to affect the properties of the proteins substantially.[17] However, their true location and significance must await further analysis by DNA sequencing and immunoelectrophoresis of protein products.

Two of the three new mutations found in this study were present in asymptomatic mothers of two patients and in one sib, and could therefore be uncommon population polymorphisms of no pathological significance. However, clinical studies suggest that penetrance is variable and apparently unaffected relatives may have histologically abnormal mitochondria.[4] In addition, age of onset of symptoms may be very variable even within a family (case 10). Neither of these parents has been investigated, and even muscle biopsy could not unequivocally exclude a mild defect in an unbiopsied muscle. There have been only two previously reported DNA studies of mitochondrial myopathy.[18 19] Although they identified population polymorphisms, they did not find any alterations which they considered to be of pathological significance. Both of these studies used total mitochondrial DNA as a probe, which may give ambiguous results. Our study has taken this a step further by using a series of probes to give more regional detail over a range of patients and has excluded deletions, insertions, or rearrangements of 60 base pairs or above from 83% of the protein reading frames (fig 1). Regional probes were not available for the remaining 17%, as this region is peculiarly resistant to cloning. The rest of the genome is probably not relevant to the defects in these particular patients.

The 13 reading frames of the mitochondrial genome encode only a small minority of the known protein subunits of the electron transport chain. The remainder are encoded by nuclear genes, and in addition there must be many regulatory genes involved in assembly. These figures suggest that most cases of mitochondrial myopathy are the result of mutations in nuclear genes and would occur sporadically or follow an autosomal recessive inheritance pattern. The majority of biochemical defects in man have such a mode of inheritance.[20] However, Wallace *et al*[21] have pointed out that the higher mutation rate of mitochondrial DNA as compared with nuclear DNA may increase the likelihood of fixation for mitochondrial mutations. If so, a mitochondrial mutation is a reasonable hypothesis in families with apparent maternal inheritance.

The patients in this study were a representative group of cases of mitochondrial myopathy occurring after the neonatal period,[1–3 22] and in no case was the diagnosis in doubt. Mitochondrial DNA extracted from white cells is almost certainly identical to that found in skeletal muscle,[23] although heteroplasmy occurs rarely in cows.[24] However, heteroplasmy does remain a theoretical possibility and might conceivably explain the distribution of affected tissues in mitochondrial myopathy and the variation in severity within a family. In this unlikely event, mitochondrial DNA from white cells might be unrepresentative and not reflect the type found in skeletal muscle.

In conclusion, these results show that large

deletions or insertions are not present in the mtDNA of a representative selection of patients with mitochondrial myopathy. If these disorders result from mutations in mitochondrial DNA these are likely to be point mutations or small structural rearrangements.

Changes in restriction fragment size explicable by such mutations were observed in three out of 10 patients. It is likely that these represent silent population polymorphisms. Identification of pathologically significant mutations in the mitochondrial genome of patients with mitochondrial myopathy will require sequence analysis of appropriate regions and biochemical studies of the corresponding protein subunit.

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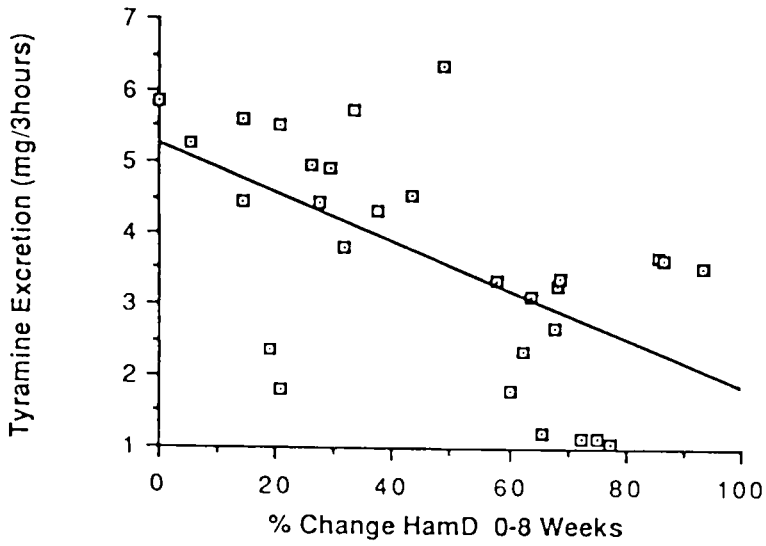

Fig 2—Relation between baseline tyramine excretion rate and improvement in HamD score.

B, responders had a tyramine excretion significantly lower than that of non-responders—2·68 (1·12) mg/3h *vs* 4·67 (1·25) mg/3h; $t = 4·57$, $p < 0·01$. There was a highly significant correlation between pretreatment excretion and both the percentage change in HamD scores ($r = 0·62$, $p = 0·005$) (fig 2) and the final HamD score ($r = 0·51$, $p = 0·003$). Both findings remained valid when "endogenicity", as measured by the Newcastle score, was partialled out. With stepwise multiple regression, the intercorrelation between tyramine excretion and the Newcastle score was −0·396. The coefficients of determination of R^2 (variance in HamD score change accounted for) with both tyramine sulphate excretion and Newcastle score was 0·360, whereas the unique contribution of tyramine excretion (R^2 change) was 0·1881 and of the Newcastle score, 0·045.

There was no significant association between pretreatment excretion and initial HamD score, weight, or age. Neither weight nor age correlated with any measure of improvement. Also, there were no significant relations between initial HamD score and percentage change, Newcastle score, or final HamD score. The Newcastle score was not a useful predictor of treatment outcome: 9 responders and 3 non-responders (criterion B) had Newcastle scores of 6 or more ($\chi^2 = 3·4722$, $p = 0·062$). There was no significant difference in mean weight between the high and low excretion groups—60·3 (5·78) kg *vs* 62·3 (10·0) kg. The response rate to clomipramine (9/15 subjects), amitriptyline (2/5), and imipramine (4/10) did not differ significantly.

Discussion

The results show that there is a highly significant relation between pretreatment tyramine sulphate excretion and response to a normal clinical dosage of tricyclic antidepressants given over an adequate period.[12] Both initial and final clinical assessments were done without knowledge of the excretion data in this group of patients; thus, tyramine metabolism seems to be an excellent predictor of response to antidepressants.

Despite belief to the contrary,[13-15] there is scant evidence that response to antidepressants depends on factors such as presence of biological symptoms.[16,17] Clinicians often decide to use tricyclic antidepressants only when such symptoms are present.[18] Some workers say that even if biological symptoms are independent of severity of depression, they may relate to outcome.[19] In some studies, particular biological symptoms have been given predictive weight while the overall clinical presentation has been assigned much less importance.[20,21]

We have not excluded the possibility that tyramine excretion may be secondary to other abnormalities in depressed patients but no obvious relation with other factors were seen (eg, weight), and we could not differentiate clinically between responders and non-responders. Neither the pre-treatment severity of depression nor the Newcastle scale were useful indicators of outcome in this group. The distribution of patients' Newcastle scores was not bimodal as early work on this scale has described,[10] but clustered near the cutoff value. Hence, the patients represented a group that clinically would be difficult to assign as endogenous or non-endogenous. Therefore, it is precisely such a group for whom a trial of antidepressants might be considered. Roth[22] has pointed out that the nature, purpose, and mathematical structure of scales devised to assist clinical diagnosis are different from those intended to aid prediction in a specific context. However, the purpose of diagnosis is usually to determine treatment and management. In the present investigation, no assumptions were made about the validity of a dualistic view of depressive disorder[23,24] and, indeed, the predictive value of tryamine sulphate excretion in this study was independent of any diagnostic assumptions. Nevertheless, the excretion results divided a group of patients, all with sufficient biological features to satisfy the criteria for RDC major depressive disorders, into tricyclic-responsive and tricyclic-non-responsive groups. Other widely used biological markers for depression are not good predictors: the dexamethasone suppression test does not predict outcome with antidepressant treatments.[25] Various indices of sleep electroencephalographic (EEG) recordings are present in depression, particularly the shortening of rapid eye movement (REM) latency[26] which may be a specific marker for endogenous depression.[27] Like the tyramine conjugation test, these sleep EEG measures are superior to clinical indices as predictors of treatment response.[28] However, the sleep EEG variables that best predict response are complex and they correctly classify only 55–69% of drug responders,[29] compared with 85–90% in the present study (depending on the criterion chosen for "response"). Furthermore, REM-latency measures may, unlike tyramine conjugation, be related to age[30] and severity of illness.[31]

In patients with unipolar depression, tyramine sulphate excretion seems to predict treatment outcome and, therefore, low output would justify a trial of tricyclic antidepressants in an individual patient. Because tyramine excretion is not affected by treatment with tricyclics,[4] it is possible that even after treatement has begun, the test may also help with the management of patients who may not have responded to lower doses, or short trials. Clearly, some patients who eventually respond show little sign of doing so for the first 4 or 5 weeks.[12,20] Some patients respond to tricyclic medication only in large daily doses.[9,32,33] Hence, low tyramine excretion in a non-responder may point to the need for either a dosage increase or a longer period on the medication.

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DUPLICATIONS OF MITOCHONDRIAL DNA IN MITOCHONDRIAL MYOPATHY

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Summary Restriction enyzme analysis was done on total cellular DNA extracted from whole blood in two patients with mitochondrial myopathy and multisystem involvement and their families. The two patients had an abnormal mitochondrial genome with a large (about 8 kb) duplication present in several tissues. Normal mitochondrial DNA (mtDNA) was also present, but within each maternal lineage the abnormal mitochondrial genome was confined to clinically affected individuals. This observation, together with the failure of extensive population surveys to identify such abnormalities of mtDNA, suggests that these mutations cause the disease.

Introduction

Although most genes are situated in the cell nucleus, an important minority are found within the mitochondria. Mitochondrial DNA (mtDNA) was first recognised 40 years ago,[1] but mitochondrial mutations have only lately been associated with disease in man.[2-4] MtDNA is probably the most completely characterised section of the genome in multicellular animals.[5] Human mtDNA has been fully sequenced[5] and is known to encode 13 proteins, including subunits of the electron transport chain and ATP synthase.[6,7] Each of the thousands of mitochondria present in every human cell usually contains 2–10 identical copies of a 16·5 kb circular genome. MtDNA is maternally inherited, the spermatozoan contributing almost no mtDNA to the conceptus.

The mitochondrial myopathies are a group of disorders characterised by abnormal mitochondria in skeletal muscle.[8] The clinical features are varied, ranging from mild muscle weakness to severe multisystem disease with central nervous system involvement.[8] Most cases are sporadic, but there is a high ratio of maternal to paternal transmission in familial cases,[8-10] indicating that some could be caused by mutations in mtDNA. Furthermore, mtDNA encodes subunits of the respiratory chain complexes which may be defective in some patients.[8]

Evidence that mitochondrial mutations are a cause of mitochondrial myopathy comes from the finding of a population of deleted mtDNA from skeletal muscle in some patients.[2-4] Because the abnormal mtDNAs were confined to muscle, and the normal mtDNAs were present in all tissues, both heteroplasmy (the existence of more than one distinct type of mtDNA in a single organism) and tissue moscaicism (different ratios of the two mitochondrial genotypes in different tissues) were apparent.

We report two patients with mitochondial myopathy and multisystem involvement in whom we have identified heteroplasmy for an abnormal mtDNA population in white cells and other tissues.

Patients and Methods

Patients

The two patients are part of a group of eighteen. The clinical picture has been reported in eleven of these patients.[11,12]

Patient 1[12] presented with diabetes mellitus and small stature at the age of 7 years. Classic clinical features of Kearns-Sayre syndrome developed,[8] with progressive visual impairment, external ophthalmoplegia, ptosis, retinal degeneration, ataxia, muscle weakness, and deafness. Muscle biopsy showed ragged red fibres diagnostic of mitochondrial myopathy. Family members were unaffected.

In patient 2 mitochondrial myopathy was diagnosed at the age of 12 years when she had a progressive ataxia, dysarthria, muscle weakness, external ophthalmoplegia, ptosis, and mental retardation.

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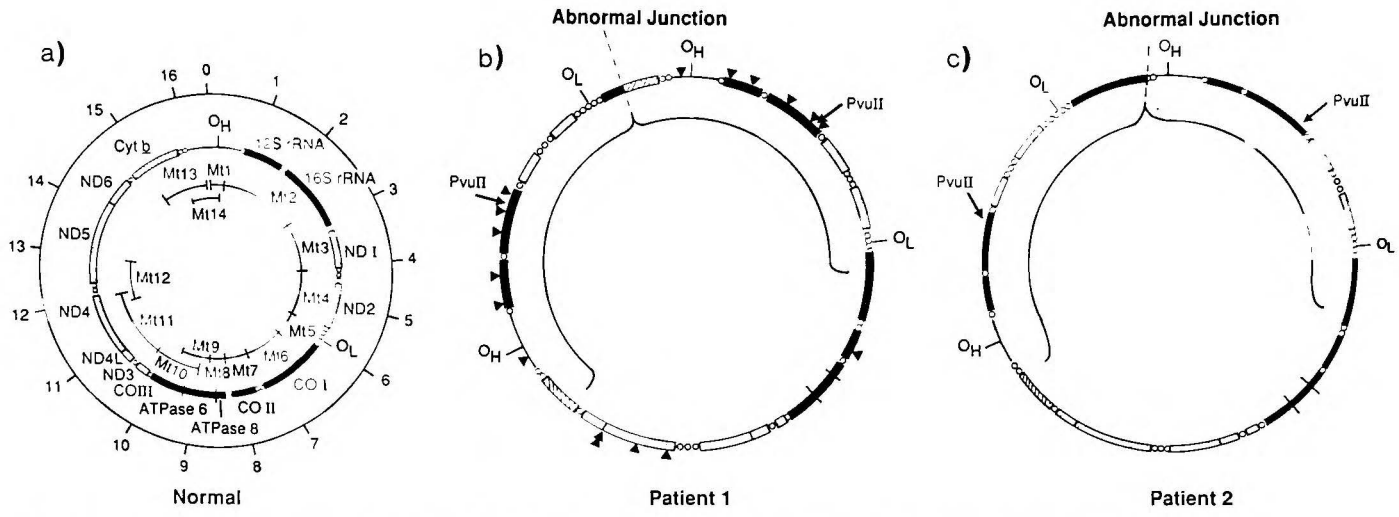

Fig 1—Schematic representation of two duplicated mitochondrial genomes in relation to known map of mtDNA.

(a) Human mitochondrial genome showing location of probes used. CO I to CO III subunits 1–3 of cytochrome C oxidase; ND 1–6 and ND 4L subunits 1–6 and 4L of NADH dehydrogenase; ATPase 6 and 8; subunits 6 and 8 of H^+ ATP synthase; Cyt b, apocytochrome b; Mt 1–14 regions correspond to probes 1–14 referred to in text; O = transfer RNAs; O_H and O_L = origins of replication of heavy and light strands; outer circle reference numbers after Anderson et al.[5] (b) Duplication found in patient 1, same scale as (a). Brackets = duplicated regions; →*Pvu* II–*Pvu* II sites; ▼*Ava* II sites; ⏬ *Bam* HI site. (c) Duplication found in patient 2.

1 year later diabetes developed and she subsequently had a stroke-like episode with transient right hemiparesis. Needle muscle biopsy early in her illness showed fat deposition and degenerating fibres on light microscopy and increased numbers of mitochondria and myofilament loss on electron microscopy.

Laboratory Methods

DNA was extracted from whole blood and Epstein-Barr-virus-transformed lymphocyte lines from all patients and their mothers and from the families of patients 1 and 2 by standard methods,[13] from lymphocyte lines and hair from patients 1 and 2,[14] and from muscle[15] in patient 1. Blood DNA was digested with restriction enzymes, electrophoresed overnight in 1% agarose gels, Southern blotted,[16] and hybridised with ^{32}P-radiolabelled[17] purified mtDNA and fourteen mitochondrial probes in bacteriophage M13 (fig 1a). Autoradiography was done overnight at −80°C with intensifying screens.

The polymerase chain raction (PCR)[18] is an enzyme method for synthesising multiple copies of a DNA segment whose ends are specified by oligonucleotide primers added to the reaction mixture. The DNA segment may be a tiny fraction of the starting material and may be added as a crude cell lysate.[19] DNA is made by repetitions of a three-stage cycle, consisting of denaturation, annealing, and extension. First, two strands of the DNA segment are separated by heating (denaturation). On cooling renaturation is impaired by an excess of the two primers which anneal to the parent strands. DNA is then synthesised along the parent strand by a heat-stable polymerase primed by the annealed oligonucleotide segment (extension). At the end of the first cycle there are thus two copies of the parent strand, at the end of the second there are four, and so on, the product of one primer acting as a template for the other. Exponential amplification of the specified fragment therefore occurs. PCR amplification[18] was done on 150 ng of digested DNA, 10 µl of crude cell lysates from granulocyte colonies[20] or bladder epithelial cells, or a quarter of the DNA extracted from a single hair with thermostable *Taq* polymerase ('Cetus'; Perkin Elmer) and the recommended buffer. Thirty cycles were used; denaturation at 93°C for 60 s, annealing at 45°C for 60 s, and extension at 72°C for 150 s in a computer-controlled alternating temperature water bath. A tenth of the product was electrophoresed on 1% agarose gel stained with ethidium bromide and photographed under ultraviolet light.

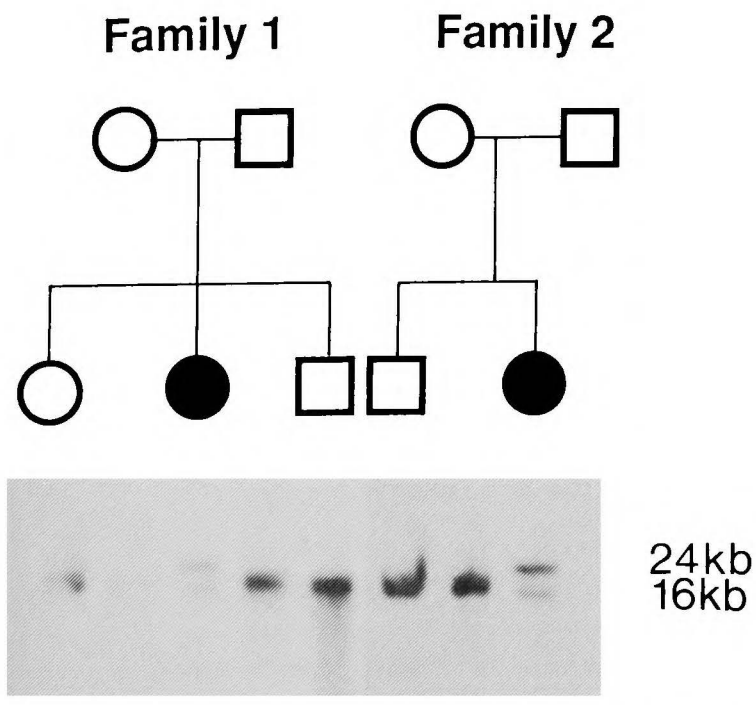

Fig 2—Restriction enzyme analysis and pedigree of 2 patients with mitochondrial myopathy and their families.

●, affected female; ○, unaffected female; □, unaffected male. (No DNA available from father in family 2.)

Results

Restriction enzyme analysis was done on total cellular DNA extracted from whole blood from the eighteen patients, and from the mothers, unaffected siblings, and one of the fathers of patients 1 and 2, probing with either purified whole human placental mtDNA or one of fourteen small fragments of HeLa cell mtDNA in bacteriophage M13 (fig 1a, 2). MtDNA contains a single restriction site for *Bam* HI which linearises the genome, and hybridisation with total mtDNA reveals a single 16·5 kb band. An extra band 8 kb larger than the normal band was present in patients 1 and 2, but not in unaffected family members, suggesting that either an insertion or a duplication was present (fig 2). Similar bands were observed with undigested DNA. No abnormalities were seen in the remaining sixteen subjects.

In patient 1 digestion with *Ava* II revealed an additional abnormal band of 4·6 kb (fig 3) which hybridised with probes 13, 14, 3, 4, and 5, suggesting that the additional DNA was mitochondrial in origin. Since these probes hybridised with portions of the genome usually distant from

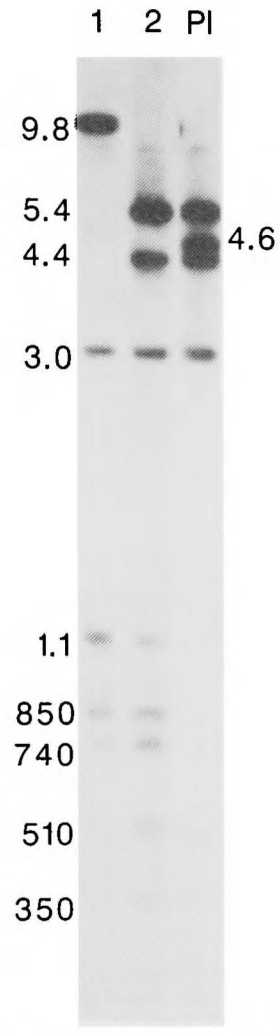

Fig 3—Restriction enzyme analysis with *Ava* II.

Hybridisation with purified mitochondrial DNA may reveal various polymorphisms, two of which are shown (morphs 1 and 2). Patient 1 was a morph 2 but with an extra 4·6 kb band. 1 = morph 1; 2 = morph 2; P1 = patient 1.

each other, this finding indicates an abnormal junction between these two regions. Furthermore, since the 4·6 kb band is shorter than the 8 kb extra DNA, the latter must contain an internal *Ava* II site. An insertion was excluded by the absence of a second abnormal fragment. Hence a tandem duplication was present, and was confirmed by the results of digestion with *Bst* NI, *Eco* RI, *Hpa* I, and *Sac* I, and hybridisation with total mtDNA.

Normal mtDNA contains a single *Pvu* II site which is situated within the region thought to be duplicated in both patients. Digestion with this enzyme and hybridisation with total mtDNA revealed, in addition to the normal 16·5 kb band, an abnormal, about 8 kb, band. This band represents the full length of the extra DNA, and suggests that the duplicated DNA contained a *Pvu* II site (figs 1b, 4). This digest was hybridised with each of probes 1–14 to localise the duplication. The results of the hybridisation suggested that the duplicated portion was situated at a position equivalent to between 15 000 and 6000 bases of the reference sequence (see fig 1) in patient 1 (fig 4a) and at between about 16 000 and 7000 bases in patient 2 (fig 4b).

The PCR was used in each case to amplify across the junctions between repeats to localise the junction more precisely. Amplification with primers COI 1 (bases 6004 to 6023 of the reference sequence)[5] and D5 complementary to bases 16 255 to 16 274 produced a 1·5 kb fragment in patient 1 and a 1·8 kb fragment in patient 2 (fig 5). PCR with this pair of primers in normal controls did not give rise to any products since the primers were too distant from each other for the amplification reaction to occur under the conditions

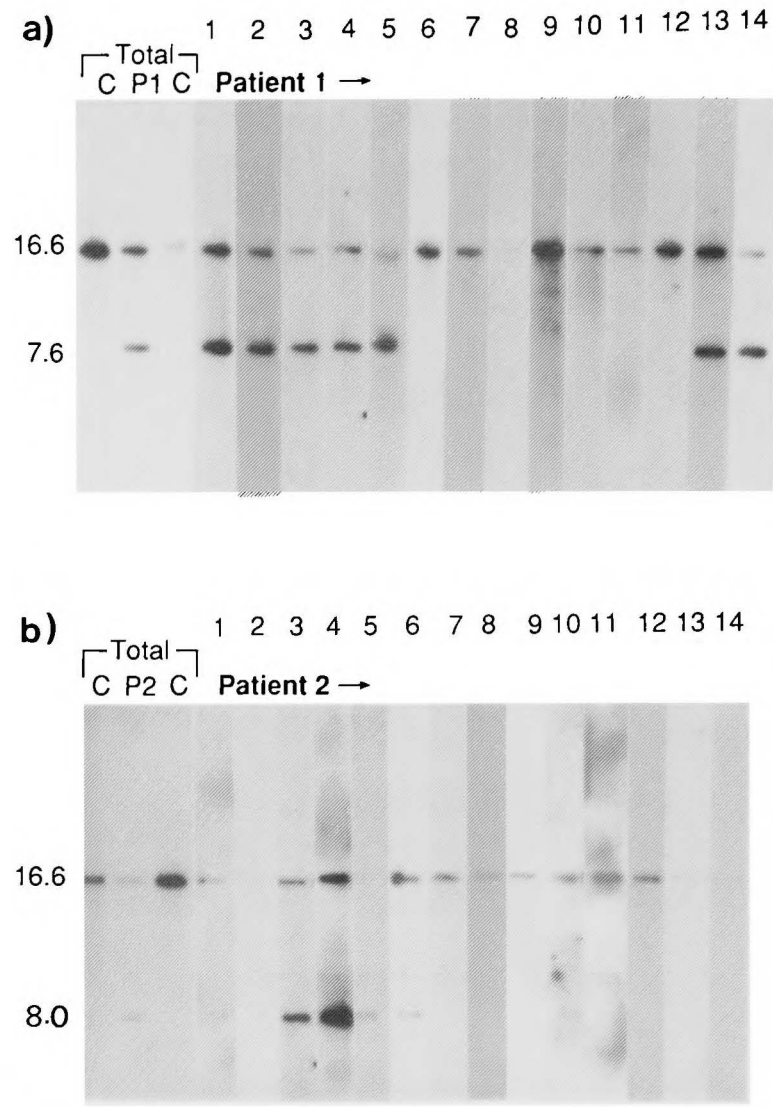

Fig 4—Position of tandem duplications in patient 1 (a) and patient 2 (b).

Patient 1, abnormal ~7·6 kb fragment hybridised with probes 13, 14, and 1–5; patient 2, abnormal ~8·0 kb fragment hybridised with probes 14 and 1–6. C = control; P1 = patient 1; P2 = patient 2; 1–14 = hybridisation with mitochondrial probes 1–14 (see fig 1); total = hybridisation with purified mitochondrial DNA. Bands are present but have not reproduced well at 16·6 kb in lane 8 (patient 1) and in lanes 2, 5, 13, and 14 (patient 2), and at 8·0 kb in lanes 1, 2, and 14 (patient 2).

used. This finding suggests that the duplications are 7·6 (patient 1) and 8·0 (patient 2) kb pairs in length. Restriction mapping of the amplified products placed the duplicated regions at between positions 6033 (*Bst* NI site present) and 6203 (*Hind* III site absent) and about 14 960–15 130 for patient 1, and 7204 (*Hpa* II site present) and 7376 (*Bst* NI site absent) and about 15 800 and 16 129 (*Kpn* I site absent) for patient 2. The precise location of the abnormal junctions can only be fully resolved by sequencing.

Amplification of DNA from whole blood of family members did not show duplicated mtDNA in any of them. In patient 1 amplification of DNA from various tissues showed that duplicated mtDNA was easily detectable in muscle, lymphocytes, and granulocytes, but not in bladder epithelial cells or hair.

Discussion

Duplications have not been shown before in human mtDNA, despite large population surveys which have included restriction enzyme digests closely similar to ours.[21 25] The finding of distinct duplications in two patients with mitochondrial myopathy with no detectable abnormal mtDNA in the unaffected family members suggests that the association with mitochondrial myopathy is causative.

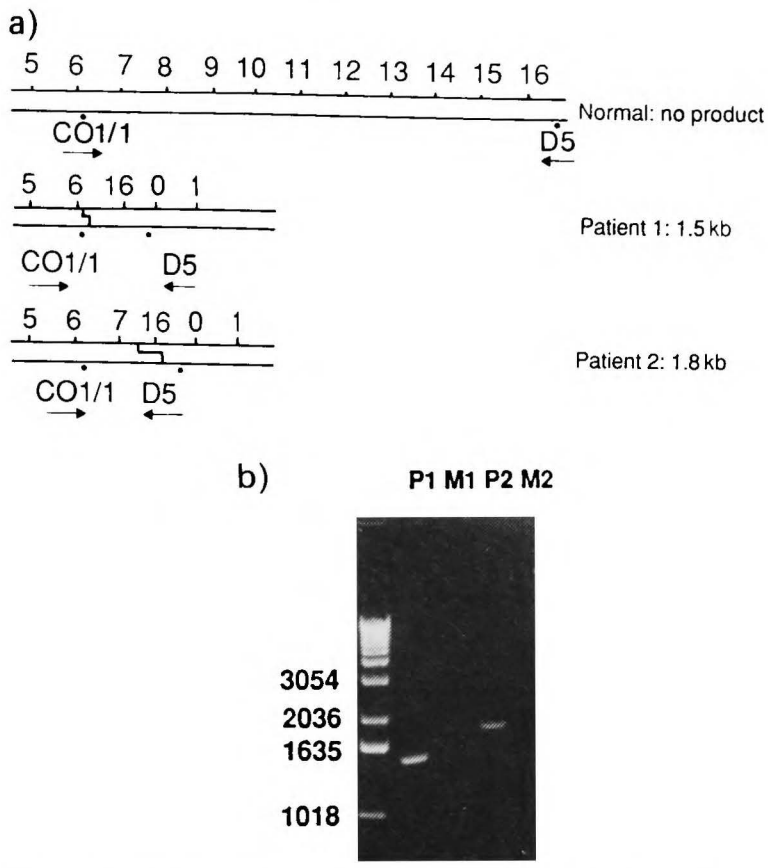

Fig 5—Amplified products of PCR with oligonucleotide primers either side of the abnormal junction: location of primers COI and D5 and expected products (a); and gel electrophoresis of products with size markers (b).

In a • = position of primers, → = direction of primers; upper bar = positions relative to reference sequence in normal controls; lower bars = approximate location of abnormal junctions. In (b) P1 = patient 1; M1 = patient 1's mother; P2 = patient 2; M2 = patient 2's mother.

Each tandem duplication included both origins of replication (O_H and O_L), both ribosomal RNA genes, part or all of four protein reading frames, and several tRNAs and all or part of the D-loop. In patient 1 the break points lay within the cytochrome oxidase I and apocytochrome b protein reading frames; in patient 2 they lay in cytochrome oxidase I and apocytochrome b, $tRNA^{Thr}$, $tRNA^{Pro}$, or the D-loop.

Because the genome is so compact any structural alterations are more likely to interrupt coding DNA than are rearrangements of nuclear genes. Although there is at least one normal copy of every gene, an interrupted gene could result in an abnormal product. For instance, cytochrome oxidase subunit I was interrupted by the cytochrome B sequence in patient 1, so that an abnormal protein might be produced, containing the cytochrome oxidase I sequence joined to an abnormal ending. Such a protein could compete with the normal product and adversely affect the function or assembly of complex II, as probably occurs in cytoplasmic male sterility in plants.[26] Alternatively, an excess of certain gene products could derange the kinetics of multimer assembly. Changes in mitochondrial transcription and protein synthesis can be analysed, and the functional consequences can be established by King and colleagues'[27] microinjection techniques.

These two duplications could have arisen by recombination of homologous sequences or by non-homologous breakage and rejoining. Circular dimers are occasionally formed during replication[28] and an approximately 8 kb deletion of one of these could give rise to the mutants we have shown. They would therefore be effectively duplicated for the portion not included in the deletion. It is noteworthy that most of the deletions described[2-4] are localised in a region similar to, but smaller than, the non-duplicated regions in our patients. Moritz and Brown[29] have shown tandem direct duplications in apparently symptom-free lizards, all of which included at least one protein reading frame or rRNA gene, and were adjacent to, or involved, the D-loop. Although recombination of mtDNA is rare, this region may be a recombination hot spot, possibly because during the early stages of replication it remains single stranded whereas the replication fork proceeds round two-thirds of the genome from O_H to O_L.

Both our patients showed heteroplasmy in mtDNA from blood, with normal and abnormal types being present in much the same proportions. This phenomenon is very rare in mammals,[2,30-32] although it may be stable in cell lines (ie, the ratio of the two genotypes remains unchanged over several generations). Furthermore, in patient 1 we also demonstrated that the abnormal mtDNA is also present in muscle, granulocytes, and lymphocytes. It was not easily detectable in her bladder epithelial cells or hair, and if present it is probably a small proportion. Mosaicism is therefore likely to be present, and the variation in proportions of abnormal mtDNA and the differing energy demands of various tissues may explain the phenotype of individual patients.

In neither patient can we be certain at what stage of development the duplication occurred: if it did not occur in the germline, its inferred presence in several tissues would suggest that it arose early in embryogenesis. If the duplication arose in the germline, then germline mosaicism is present and the mothers of both patients may produce further affected offspring. Germline mosaicism for nuclear genes is known to occur in man;[33] germline mosaicism for mtDNA may be present in normal cows,[34] but has not been reported in man. The possibility of germline mosaicism clearly poses a considerable problem for genetic counselling. PCR enabled accurate restriction mapping of the abnormal junction and may be useful in rapid non-invasive diagnosis of relatives at risk.

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PRACTICAL USE OF DUPLEX DOPPLER ANALYSIS OF THE RENAL VASCULATURE IN CRITICALLY ILL PATIENTS*

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Summary Duplex doppler examination of blood flow in renal interlobar arteries was analysed in twelve critically ill patients before and during low-dose dopamine infusion (2 μg/kg/min). Renal vasodilatation and increased blood flow were observed with dopamine, confirming results with indirect or invasive techniques. The doppler ultrasound technique is entirely non-invasive, is simple and quick to carry out, provides instant results, and will allow tailoring of inotropic support in critically ill patients to provide optimum renal blood flow.

Introduction

DOPAMINE, a precursor of noradrenaline, has a vasodilator effect on the renal vasculature[1,2] and is commonly used in low doses (1·5–2·5 μg/kg/min) in the management of critically ill patients with oliguria in intensive-care units (ICU). The use of such treatment was suggested by direct measurements of renal blood flow and renal vascular resistance in animal models, and the observed rises in effective renal plasma flow derived from para-aminohippurate clearance studies in man.[1-3] These studies concluded that the renal effects of dopamine could be achieved independently of changes in systemic haemodynamics. The findings were confirmed in man by direct measurement of renal venous blood flow before and during dopamine infusion by means of a local thermodilution technique.[4]

The development of duplex doppler sonography has allowed the non-invasive study of blood flow patterns in deep abdominal and pelvic vessels.[5,6] The acquisition of doppler information is combined with real-time update of the B-scan image, allowing accurate localisation of the volume of tissue containing the blood vessel giving the doppler information.

The purpose of this study was to establish whether duplex doppler ultrasound has a practical role in the management of critically ill patients in the ICU. We achieved this aim by confirming the effects of low-dose dopamine on the renal vasculature in real clinical situations, using this completely non-invasive method to measure changes in renal arterial blood flow and renovascular resistance directly.

Patients and Methods

We studied patients for whom a clinical decision to start a renovascular dopamine infusion had been made by ICU staff. We excluded patients with cardiovascular instability, rhythm disturbance, concurrent use or addition of other inotropes or vasodilator agents, and changes in ventilator settings (particularly the level of positive end-expiratory pressure) during or within an hour of the start of each study. Twelve patients were studied. Eleven were being ventilated; all had normal renal function as judged by serum creatinine.

The patient's right or left kidney was selected for study depending on ease of access. Duplex doppler examinations were carried out with a Diasonics DRF 400 ultrasound scanner with a 3·5 MHz probe. Spectral analysis was by a fast Fourier transform system incorporated in the DRF 400, analysing signal periods of 20 ms. The kidney was visualised in the longitudinal plane by real-time scanning, and the renal hilum identified. An interlobar artery at the upper or lower pole was then selected for study and doppler spectra were recorded. Two or more readings were taken from the same artery to ensure reproducibility. Each set of readings consisted of up to six consecutive cardiac cycles. All recordings were made in expiration. Dopamine infusion was then started at a rate of

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