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# Molecular Basis of Mitochondrial DNA Disease

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Mitochondrial ATP production via oxidative phosphorylation (OXPHOS) is essential for normal function and maintenance of human organ systems. Since OXPHOS biogenesis depends on both nuclear- and mitochondrial-encoded gene products, mutations in both genomes can result in impaired electron transport and ATP synthesis, thus causing tissue dysfunction and, ultimately, human disease. Over 30 mitochondrial DNA (mtDNA) point mutations and over 100 mtDNA rearrangements have now been identified as etiological factors in human disease. Because of the unique characteristics of mtDNA genetics, genotype/phenotype associations are often complex and disease expression can be influenced by a number of factors, including the presence of nuclear modifying or susceptibility alleles. Accordingly, these mutations result in an extraordinarily broad spectrum of clinical phenotypes ranging from systemic, lethal pediatric disease to late-onset, tissue-specific neurodegenerative disorders. In spite of its complexity, an understanding of the molecular basis of mitochondrial DNA disease will be essential as the first step toward rationale and permanent curative therapy.

**KEY WORDS:** Mitochondrial DNA; oxidative phosphorylation; human disease; base substitutions; rearrangements; genotype; phenotype; missense; transfer RNA.

## OXIDATIVE PHOSPHORYLATION (OXPHOS)

Cellular ATP is generated in mammalian cells via the extraordinarily complex oxidative phosphorylation (OXPHOS) pathway. OXPHOS is composed of five multi-subunit enzyme complexes within the mitochondrial inner membrane. Complexes I–IV are components of the electron transport chain which oxidizes primarily NADH and $FADH_2$ and passes the resulting electrons to the terminal acceptor oxygen which is reduced to water. During this process, protons are concurrently pumped out of the mitochondrial matrix and into the intermembrane space, generating a capacitance across the inner mitochondrial membrane. This potential energy, in the form of an electrochemical gradient, is utilized by Complex V, the ATP synthase, to condense matrix ADP and inorganic phosphate, thus forming ATP. Matrix ATP is then exchanged across the inner membrane by the adenine nucleotide translocator (ANT).

The biogenesis of OXPHOS is similarly complicated as both nuclear genes (nDNA) and mitochondrial genes (mtDNA) contribute polypeptides to Complexes I, III, IV, and V (Shoffner and Wallace, 1990). Overall, the nDNA provides greater than 65 OXPHOS gene products, and the mitochondrial genome provides 13 OXPHOS polypeptides, each of which require mitochondrially encoded rRNAs (12S and 16S) and tRNAs (22 total) for their translation. Complex I (NADH : ubiquinone oxidoreductase) incorporates seven (ND1, 2, 3, 4, 4L, 5, and 6) mtDNA gene products, Complex III (ubiquinol: cytochrome *c* oxidoreductase) incorporates one (cytochrome *b*), Complex IV (cytochrome *c* oxidoreductase) incorporates three (COI, II, and III), and Complex V incorporates two (ATPase 6 and 8) mtDNA gene products. Thus, OXPHOS biogenesis requires at least 100 gene products and is under dual genetic control. Mutations in OXPHOS

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genes of either system can impair ATP synthesis and ultimately cause tissue dysfunction.

## MITOCHONDRIAL GENETICS

A typical human cell contains many mitochondria, each with multiple copies of the 16,569 nucleotide pair (np) (Anderson *et al.*, 1981), circular mitochondrial chromosome. The high copy number of mtDNA and the cytoplasmic location of the mitochondria contribute to a unique genetic system hallmarked by five principles (Wallace, 1992). First, mitochondria are maternally inherited. Heritable pathogenic mtDNA mutations can only be transmitted from mother to offspring; thus, pedigrees exhibit maternal transmission of the clinical phenotype. Second, mitochondria undergo replicative segregation at cell division. Because there are typically 10–100 mtDNAs per organelle, a mixture of mutant and normal mtDNAs (heteroplasmy) is produced when a mutation occurs. These genotypes partition randomly at cell division into daughter cells and, depending in part on the timing of the mutagenic event, it is possible for different tissues to harbor different percentages of a deleterious mutation. Over time, the proportion of mtDNAs can drift to 100% mutant or normal mtDNAs (homoplasmy). Third, pathogenic mtDNA mutations are expressed according to a threshold effect. Certain tissues rely on mitochondrial ATP to a greater extent than do other tissues. The central nervous system (notably the brain and optic nerve), skeletal and cardiac muscle, liver, kidney, and pancreatic islet cells are particularly reliant on oxidative metabolism. Thus, three factors interact to determine the severity of a pathological mtDNA mutation: the nature of the mutation, the degree of heteroplasmy, and the oxidative requirements of the tissue. Fourth, the ATP-generating capacity of a tissue declines with age. Mitochondrial respiration studies in skeletal muscle (Trounce *et al.*, 1989) and liver (Yen *et al.*, 1989) mitochondria have shown that mitochondrial $O_2$ utilization decreases normally as a function of age. Provocatively, this attenuation of ATP synthesis corresponds to an age-related increase in somatic mtDNA damage in post-mitotic tissue (Linnane *et al.*, 1989; Cortopassi and Arnheim, 1990; Cortopassi *et al.*, 1992; Corral-Debrinski *et al.*, 1992a, b). This natural decline in ATP generating capacity may facilitate disease expression when coupled with an inherited OXPHOS mutation, particularly in the case of subtle mtDNA mutations which are only expressed in late life. Fifth, mtDNA genes have a mutation rate 10–17 times higher than nDNA genes (Neckelmann *et al.*, 1987; Wallace *et al.*, 1987). This high mutation rate may be due to one or more of the following factors: a high concentration of oxygen radicals at the mitochondrial inner membrane, the lack of efficient mtDNA repair mechanisms, and/or the absence of a DNA-coating protein such as histone. Because greater than 90% of the mitochondrial genome codes for essential OXPHOS polypeptides or structural RNAs, mutations in the mtDNA have a significant probability of impairing ATP production.

## PATHOGENIC mtDNA MUTATIONS

### Nucleotide Substitutions

Disease-causing nucleotide substitutions have been found in mtDNA protein coding, tRNA, and rRNA genes (Tables I and II). Diseases involving mtDNA nucleotide substitutions represent a broad array of clinical phenotypes, but typically feature central nervous system involvement.

#### *Missense Mutations*

Perhaps the most well-studied mtDNA nucleotide substitution disease is Leber's hereditary optic neuropathy (LHON). LHON is a disease distinguished by the rapid, painless, bilateral loss of central vision due to optic nerve atrophy (Newman *et al.*, 1991). LHON pedigree analysis reveals three characteristic features of disease transmission. First, consistent with the cytoplasmic transmission of the mtDNA, LHON is maternally inherited. Second, sporadic cases are not uncommon and penetrance within families is variable, since not all homoplasmic individuals go blind. Third, young adult males are more commonly affected than females, with 80% of Caucasian patients and 60% of Japanese patients being male (Newman *et al.*, 1991). The incomplete penetrance and male bias suggest that, although mtDNA mutations are the major etiological factors in LHON, modulating factors must influence the risk for disease expression. These factors are presently ill defined, but could include both genetic (nuclear DNA mutations or additional mtDNA mutations) or nongenetic (physiological or environmental) influences.

**Table I.** Pathogenic mtDNA Missense Mutations[a]

| Nucleotide position/gene | Disease[b] | Nucleotide change | Amino acid change | Amino acid conservation[c] | Found in controls | Heteroplasmic | Reference |
|---|---|---|---|---|---|---|---|
| 14459/ND6 | LHON | G to A | A to V | M | – | + | *d* |
| 4160/ND1 | | T to C | L to P | H | – | – | *e* |
| 11778/ND4 | | G to A | R to H | H | – | + | *f* |
| 3460/ND1 | | G to A | A to T | M | – | + | *g* |
| 14484/ND6 | | T to C | M to V | M | – | + | *h* |
| 13730/ND5 | | G to A | G to E | M | – | + | *i* |
| 15257/Cytb | | G to A | D to N | H | + | – | *j* |
| 9438/COI | | G to A | G to S | H | – | – | *k* |
| 9804/COI | | G to A | A to T | H | – | – | *l* |
| 5244/ND2 | | G to A | G to S | H | – | + | *m* |
| 7444/COI | | G to A | Term to K | na | + | – | *n* |
| 3394/ND1 | | T to C | Y to H | H | + | – | *o* |
| 15812/Cytb | | G to A | V to M | M | + | – | *p* |
| 13708/ND5 | | G to A | A to T | M | + | – | *q* |
| 4917/ND2 | | A to G | D to N | H | + | – | *r* |
| 4216/ND1 | | T to C | Y to H | P | + | – | *s* |
| 3397/ND1 | ADPD | A to G | M to V | H | + | – | *t* |
| 8993/ATP6 | NARP/Leigh's | T to G | L to R | H | – | + | *u* |
| | | T to C | L to P | H | – | + | *v* |

[a] LHON mutations are listed in estimated order of severity from most severe to least. Mutations at nps 9438, 9804, and 13730 are newly reported LHON variants; thus, their position in the table is arbitrary at this time.

[b] LHON = Leber's hereditary optic neuropathy; ADPD = Alzheimer's disease and Parkinson's disease; NARP/Leigh's = neurogenic muscle weakness, ataxia, and retinitis pigmentosa/Leigh's disease.

[c] H = highly conserved; M = moderately conserved; P = poorly conserved; na = not applicable.

[d] Wallace *et al.*, 1993a; Jun *et al.*, 1994; [e] Howell *et al.*, 1991b; [f] Wallace *et al.*, 1988a; Newman *et al.*, 1991; [g] Huoponen *et al.*, 1991; Howell *et al.*, 1991a; [h] Johns *et al.*, 1992; Johns *et al.*, 1993a; [i] Howell *et al.*, 1993a; [j] Johns and Neufeld, 1993; Brown *et al.*, 1992a; Howell *et al.*, 1993b; [k] Johns and Neufeld, 1993; [l] Johns and Neufeld, 1993; [m] Brown *et al.*, 1992a; [n] Brown *et al.*, 1992c; [o] Brown *et al.*, 1992b; [p] Brown *et al.*, 1992a; [q] Johns and Berman, 1991; Brown *et al.*, 1992a, b; [r] Johns and Berman, 1991; [s] Johns and Berman, 1991; [t] Shoffner *et al.*, 1993; [u] Shoffner *et al.*, 1992; Tatuch *et al.*, 1992; [v] deVries *et al.*, 1993.

LHON has proven to be a genetically heterogeneous disease as 16 missense mutations have been associated with the disease-to-date (Table I). Three of these at np 3460, 11778, and 14484 are generally agreed to be primary causes of LHON. Furthermore, mitochondrial genomes harboring a mutation at np 14459 or a combination of mutations at np 14484 and np 4160 not only cause LHON but are also associated with additional neurological disease. Eight other mutations are also associated with LHON. A mutation at np 15257 may be the primary cause of LHON in some patients while mutations at nps 3394, 4216, 4917, 5244, 7444, 13708, 15257, and 15812 appear to be secondary. Finally, three additional rare mutations have been proposed as primary causes of LHON at nps 9438, 9804, and 13730, but these observations need to be substantiated.

The three Complex I mutations at np 3460 in the ND 1 gene (Huoponen *et al.*, 1991; Howell *et al.*, 1991a), 11778 in the ND4 gene (Wallace *et al.*, 1988a), and 14484 in the ND 6 gene (Johns *et al.*, 1992) are the most common causes of LHON (Table I). With the possible exception of the 14484 mutation, these variants are capable of potentiating blindness by themselves as autonomous etiological entities. The genetic characteristics of these variants indicate their relative severity. Each has been found in either the homoplasmic or heteroplasmic state in patients, has only been found in patients and never in controls, alters a moderately to highly conserved amino acid in an evolutionarily constrained polypeptide domain, has occurred on different mtDNA backgrounds and each time resulted in disease, and has a relatively high penetrance in LHON families.

Although the np 14484 ND6 mutation is clearly a primary LHON mutation, it may be less deleterious than the np 3460 ND1 or 11778 ND4 mutations. Unlike the ND1 and ND4 mutations which cause more radical amino acid substitutions at more highly conserved residues, this base change results in a conservative (methionine to valine) replacement of a moderately conserved amino acid. Visual improvement

**Table II.** Pathogenic mtDNA, rRNA, and tRNA Mutations[a]

| Nucleotide position/gene | Disease[b] | Nucleotide change | Nucleotide conservation[c] | Found in controls | Heteroplasmic | Reference |
|---|---|---|---|---|---|---|
| | | | rRNA mutations | | | |
| 1555/12S rRNA | DEAF | A to G | H | – | – | *d* |
| 721/12S rRNA | ADPD | T to C | M | – | – | *e* |
| 3196/16S rRNA | | G to A | nd | – | + | *f* |
| | | | tRNA mutations | | | |
| 3243/tRNA$^{Leu(UUR)}$ | MELAS | A to G | H | – | + | *g* |
| | DMDF | A to G | H | – | + | *h* |
| 3271/tRNA$^{Leu(UUR)}$ | MELAS | T to C | M | – | + | *i* |
| 8344/tRNA$^{Lys}$ | MERRF | A to G | H | – | + | *j* |
| 8356/tRNA$^{Lys}$ | | T to C | M | – | + | *k* |
| 3260/tRNA$^{Leu(UUR)}$ | HCM | A to G | H | – | + | *l* |
| 3303/tRNA$^{Leu(UUR)}$ | | A to G | nd | nd | + | *m* |
| 9997/tRNA$^{Gly}$ | | A to G | H | – | + | *n* |
| 3250/tRNA$^{Leu(UUR)}$ | MM | T to C | P | – | + | *o* |
| 3302/tRNA$^{Leu(UUR)}$ | | A to G | M | – | + | *p* |
| 15990/tRNA$^{Pro}$ | | G to A | H | – | + | *q* |
| 4269/tRNA$^{Ile}$ | FICP | A to G | P | – | + | *r* |
| 4317/tRNA$^{Ile}$ | | A to G | nd | – | nd | *s* |
| 4336/tRNA$^{Gln}$ | ADPD | A to G | M | + | – | *t* |
| 15923/tRNA$^{Thr}$ | LIMM | A to G | nd | – | – | *u* |

[a] Mutations listed by disease.

[b] DEAF = maternally inherited sensorineural deafness; ADPD = Alzheimer's disease and Parkinson's disease; MELAS = mitochondrial encephalomyopathy, lactic acidosis, and stroke-like symptoms; DMDF = diabetes mellitus and deafness; MERRF = myoclonic epilepsy and ragged-red fiber disease; HCM = hypertrophic cardiomyopathy and myopathy; MM = mitochondrial myopathy; FICP = fatal infantile cardiomyopathy plus; LIMM = lethal infantile mitochondrial myopathy.

[c] H = highly conserved; M = moderately conserved; P = poorly conserved; nd = no data.

[d] Prezant *et al.*, 1993; [e] Brown *et al.*, 1994; [f] Shoffner *et al.*, 1993; [g] Goto *et al.*, 1990; [h] van den Ouweland *et al.*, 1992; Gerbitz *et al.*, 1993; [i] Goto *et al.*, 1991; [j] Shoffner *et al.*, 1990; [k] Silvestri *et al.*, 1992; Zeviani *et al.*, 1993; [l] Zeviani *et al.*, 1991; [m] Silvestri *et al.*, 1993; [n] Merante *et al.*, 1993; [o] Goto *et al.*, 1992; [p] Bindoff *et al.*, 1993; Shoffner *et al.*, 1993; [q] Moraes *et al.*, 1993; [r] Taniike *et al.*, 1992; [s] Tanaka *et al.*, 1990; [t] Shoffner *et al.*, 1993; [u] Yoon *et al.*, 1991; Brown *et al.*, 1992d.

occurs more frequently in 14484-positive patients (approximately 35% of patients) than in 3460-positive (approximately 22%) or 11778-positive patients (4%) (Johns *et al.*, 1993a). Finally, 3460- and 11778-positive patients only occasionally harbor additional lower-risk or "secondary" LHON mutations while 14484-positive patients frequently harbor secondary LHON mutations including the nps 13708, 15257, 3394, or 7444 mutations (Johns *et al.*, 1992; Brown *et al.*, 1992a, b; Mackey and Howell, 1992). Therefore, the np 14484 mutation may be a mildly deleterious missense mutation, the penetrance of which is enhanced by other subtle mtDNA mutations.

The np 15257 mutation in the cytochrome *b* gene appears to bridge the gap between the primary LHON mutations and the secondary supporting LHON mutations. Consistent with other primary LHON mutations, np 15257 replaces a conserved aspartate with an asparagine in an evolutionarily constrained region of cytochrome *b* believed to be essential for heme binding (Johns and Neufeld, 1991; Brown *et al.*, 1992a), has been found in more than 25 LHON families (Johns and Neufeld, 1991; Brown *et al.*, 1992a; Howell *et al.*, 1993b; Johns *et al.*, 1993b), has occurred on different LHON mtDNA haplotypes (Brown *et al.*, 1992a; Howell *et al.*, 1993b; unpublished data, this laboratory), and has been found in patients who lack other known primary LHON mutations (Howell *et al.*, 1993b; unpublished data, this laboratory). In addition to these features of primary LHON mutations, this variant has been reported to be associated with increased incidence of spinal cord and peripheral neurological symptoms in LHON

patients (Johns *et al.*, 1993b) as well as retinal maculopathy (Heher and Johns, 1993). However, like the secondary LHON mutations, this variant has been detected in at least 2/500 (0.4%) of controls (Brown *et al.*, 1992a, unpublished data, this laboratory) and is sometimes associated with a primary LHON mutation, most commonly the np 14484 ND 6 mutation (Heher and Johns, 1993; Johns *et al.*, 1993a; Brown *et al.*, 1992a), but also np 11778 (Huoponen *et al.*, 1993). When coupled with the np 11778 LHON mutation, np 15257 increased the penetrance to 44% in a np 11778-positive family (Huoponen *et al.*, 1993). Biochemical analysis and more genetic data such as the complete sequences of 15257-positive patient and control chromosomes may help to clarify the pathogenicity of this missense mutation.

The seven secondary LHON missense mutations at nps 3394, 4216, 4917, 5244, 7444, 13708, and 15812 have been found at increased frequencies in LHON patients, but are almost always linked to primary LHON mutations. Hence, they may increase the risk of expressing LHON (Johns and Berman, 1991; Brown *et al.*, 1992a, b, c; Johns *et al.*, 1993b; Johns and Neufeld, 1991). For example, the np 7444 G to A mutation is unique in that it extends the COI polypeptide by three amino acids, an alteration detectable by SDS-PAGE gel electrophoresis. This variant has been found in at least two LHON patients (9%, Brown *et al.*, 1992c) and in at least one confirmed control (Brown *et al.*, 1992c). In one patient, np 7444 occurred with the np 3460 mutation (Brown *et al.*, 1992c), and in the other with the np 14484 (M. D. Brown, A. Torroni, and D. C. Wallace, unpublished data). Thus, 7444-positive patients may not express LHON unless they harbor another primary mutation. The np 3394 mutation changes a highly conserved tyrosine in ND1 to a methionine, and is common in French Canadian LHON patients. These individuals also harbor the np 14484 mutation (Brown *et al.*, 1992b). The remaining secondary LHON mutations show much less consistent associations.

Other mtDNA missense mutations cause more severe human diseases. A missense mutation at np 14459 of the ND6 gene is believed to cause not only LHON, but also childhood dystonia associated with bilateral basal ganglia degeneration (LHON + dystonia) (Wallace *et al.*, 1993a; Jun *et al.*, 1994). This mutation was found to be heteroplasmic in a large, five-generation Hispanic family expressing maternally inherited LHON and/or early-onset dystonia (Fig. 2). Thirteen of the 22 affected family members manifested the severe neurological disease, eight expressed LHON, and one individual presented with both LHON and dystonia (Novotny *et al.*, 1986). Complete sequence analysis of the proband's mtDNA revealed 40 sequence changes relative to the standard Cambridge sequence. These include the heteroplasmic np 14459 mutation, as well as three polymorphic variants at nps 10398, 10400, and 5178 which identified the mtDNA as related to the Native American mtDNAs of haplogroup D (see Torroni and Wallace, this issue). The np 14459 G to A substitution changes a moderately conserved alanine to a valine in a conserved region of the ND6 polypeptide, and is only eight amino acids away from the methionine-to-valine replacement caused by the np 14484 mutation. The np 14459 mutation was not found in 310 normal controls of various ethnic groups, nor was it found in 38 haplotype-matched individuals of Native American haplogroup D (Jun *et al.*, 1994; Torroni *et al.*, 1992, 1993; Torroni and Wallace, this issue). Thus, the np 14459 mutation is unique to this pedigree and could account for the range of phenotypes observed in this LHON + dystonia family by virtue of meiotic and mitotic replicative segregation.

Another pair of heteroplasmic missense mutations, a T-to-G transversion or a T-to-C transition at np 8993 of the ATPase6 gene, also results in highly variable phenotypes within families. In one pedigree, the phenotype of the T-to-G mutation has been described as neurogenic muscle weakness, ataxia, and retinitis pigmentosa (NARP) but in most pedigrees, these mutations present as Leigh's disease (Holt *et al.*, 1990; Tatuch *et al.*, 1992; Shoffner *et al.*, 1992; deVries *et al.*, 1993). Both mutations cause radical amino acid substitutions of a highly conserved leucine residue, the T-to-G mutation creating an arginine codon and the T-to-C mutation creating a proline codon. Amino acid substitutions at this position appear to inhibit ATP production by blocking the proton channel of the ATPase (Tatuch *et al.*, 1992; Trounce *et al.*, 1994). In most pedigrees, the severity of clinical symptoms correlates well with the proportion of mutated mtDNAs found in patient tissues. Leigh's disease is common when very high percentages (> 90%) of one of the ATPase6 mutations is found. As the proportion of mutant mtDNAs drops below approximately 90%, NARP becomes a common clinical manifestation, and if the percentage of mutant mtDNAs falls between 70 and 80% mutant, peripheral retinopathy is common.

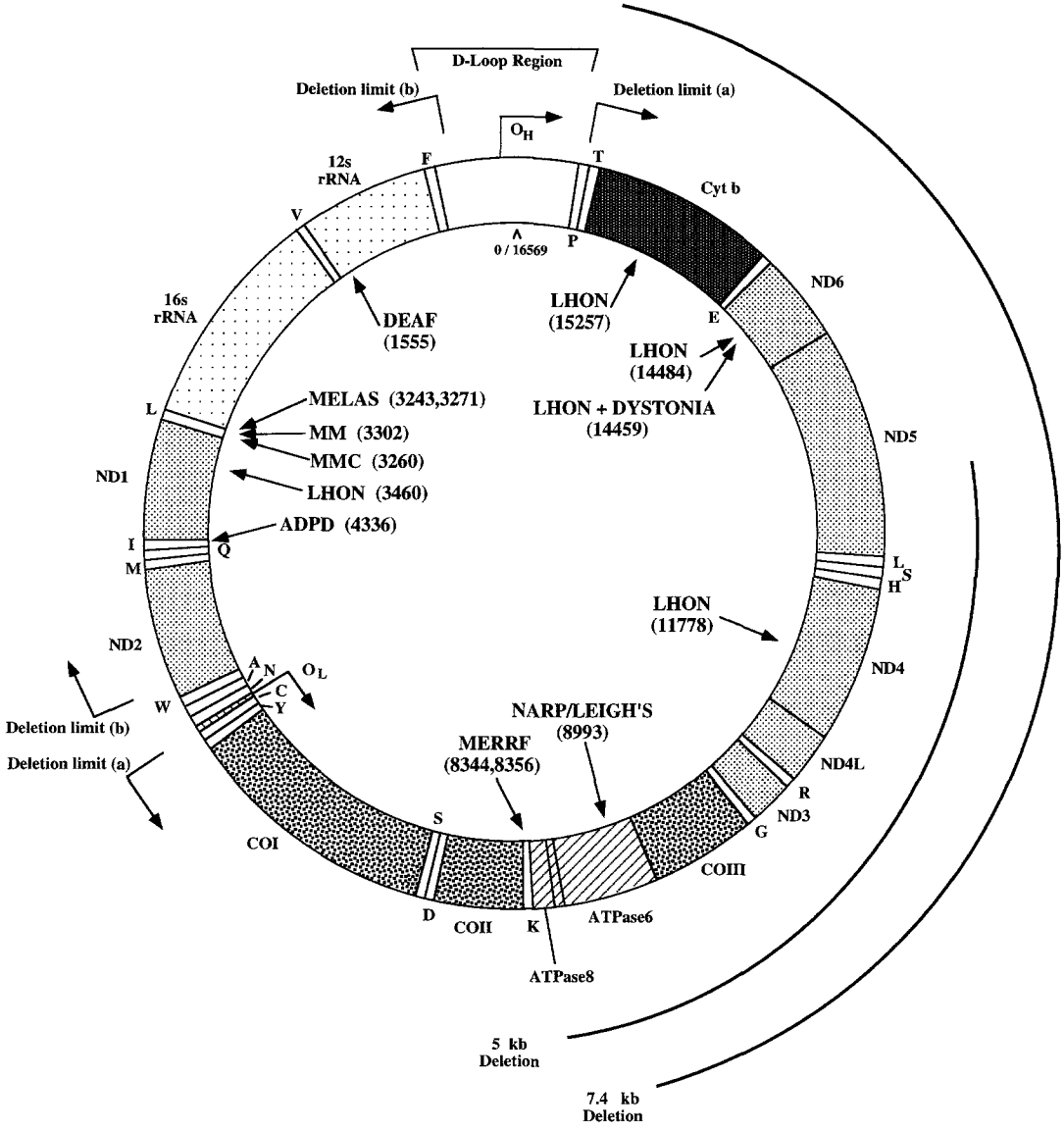


**Fig. 1.** Map of the human mitochondrial DNA showing positions of well-characterized mtDNA point mutations (inside of circular chromosome) and deletions (outside of circular chromosome). Origins of DNA replication for the heavy-strand ($O_H$) and the light-strand ($O_L$) are indicated. Note that deletion limits sparing $O_H$ and $O_L$ are indicated. See text and Tables I and II for disease designations.

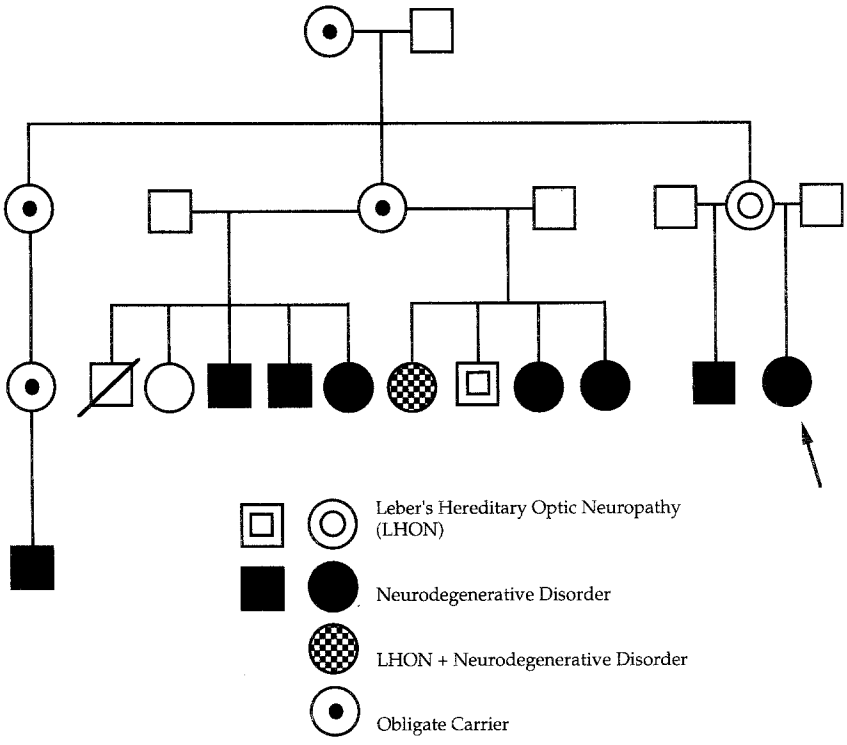


**Fig. 2.** Partial LHON + dystonia pedigree showing maternal inheritance and the spectrum of clinical symptoms resulting from the heteroplasmic np 14459 mutation. Proband is indicated by arrow.

The differences in the clinical phenotypes of the various mtDNA missense mutations (LHON, LHON and dystonia, and NARP/Leigh's disease), underscores the clinical variability of mtDNA disease. Some mutations, like those resulting in LHON, have a tissue-specific clinical phenotype in some cases caused by multiple mutations, while others like LHON + dystonia and NARP/Leigh's disease represent diseases of a broader clinical spectrum. Such variability in genotype/phenotype associations is not easily explained by a single factor (e.g., heteroplasmy) and serves to underscore the complexity of mitochondrial genetics with respect to OXPHOS impairment and human disease.

*Transfer RNA (tRNA) Mutations*

Fourteen mtDNA tRNA mutations have been associated with maternally inherited human disease (Table II). Transfer RNA mutations typically result in severe mitochondrial myopathies hallmarked by "ragged red" skeletal muscle fibers upon Gomori trichrome staining and the accumulation of structurally abnormal mitochondria in muscle (Wallace, 1992). Almost invariably, pathogenic tRNA mutations are heteroplasmic, alter moderate or highly conserved nucleotides, are not found in control populations, and are associated with detectable OXPHOS enzyme deficiencies. Significant clinical heterogeneity within and between families with a particular tRNA disorder is common, a phenomenon only partly explained by heteroplasmy. Mutations in tRNAs exemplify the threshold effect with respect to replicative segregation as individuals may not exhibit clinical signs unless the proportion of mutant mtDNA exceeds 80–90%. Three well-characterized mitochondrial myopathies due to mtDNA tRNA mutations are myoclonic epilepsy and ragged red fiber (MERRF) disease; mitochondrial encephalomyopathy, lactic acidosis and stroke-like symptoms (MELAS); and maternally inherited myopathy and cardiomyopathy (MMC).

Sequence analysis of patient mtDNAs has shown that MERRF is the result of allelic point mutations in the $tRNA^{Lys}$ gene. A G-to-A transition at np 8344 (Shoffner *et al.*, 1990) alters a conserved nucleotide

in the TΨC loop and accounts for 80–90% of MERRF pedigrees, while a T-to-C transition at np 8356 (Silvestri *et al.*, 1992; Zeviani *et al.*, 1993) destabilizes a highly conserved base pair in the TΨC stem of $tRNA^{Lys}$ and has been found in np 8344-negative patients. Both mutations are heteroplasmic and have not been found in unaffected individuals.

Pathogenic tRNA mutations such as the MERRF mutations typically impair the translation of mitochondrial mRNAs and result in pleiotropic OXPHOS defects. This is true in np 8344-positive patients as analysis of mitochondrial translation products in lymphoblasts (Wallace *et al.*, 1986), skeletal muscle (Noer *et al.*, 1991), myoblasts (Boulet *et al.*, 1992), and cybrid constructs derived from skeletal muscle (Chomyn *et al.*, 1991) indicate a translational defect. The disruption of normal protein synthesis is reflected in the reduced activities of those OXPHOS enzyme complexes which contain the highest number of mitochondrially encoded polypeptides, Complexes I and IV (Wallace *et al.*, 1988b; Yoneda *et al.*, 1990).

Among 8344-positive MERRF patients, the severity of clinical symptoms is roughly proportional to the severity of the OXPHOS defect when age is accounted for (Shoffner *et al.*, 1990). Because OXPHOS capacity declines with age, two individuals with identical mtDNA genotypes may express differing clinical signs if they are of different ages. For example, pedigree members under the age of 20 harboring 80% mutant mtDNAs may be asymptomatic while individuals with the same genotype over the age of 60 may suffer severe, multi-system neurological disease. Thus, older individuals have a lower threshold for OXPHOS dysfunction and therefore cannot tolerate significant OXPHOS impairment without clinical consequences. Heteroplasmy coupled with the age-related diminution of OXPHOS capacity of affected tissues can thus explain not only clinical variability seen within and between MERRF pedigrees, but also the progressive course of the disease.

Two $tRNA^{Leu(UUR)}$ point mutations account for the majority of MELAS patients. An A-to-G transition at np 3243 has been found in approximately 80% of MELAS patients (Goto *et al.*, 1990), while a T-to-C transition at np 3271 (Goto *et al.*, 1991) was found in an additional 7.5% of np 3243-negative patients. The np 3243 mutation alters a highly conserved nucleotide within the dihydrouridine loop, and the np 3271 mutation disrupts a conserved base pair in the tRNA anticodon stem. In both cases, the mutations are heteroplasmic and are not found in controls. Like MERRF, heteroplasmy contributes to the considerable variability of clinical signs observed within and between MELAS pedigrees. Unlike the MERRF variants, however, the np 3243 mutation can result in a number of different clinical syndromes in addition to MELAS. These include diabetes mellitus and deafness (van den Ouwerland *et al.*, 1992; Gerbitz *et al.*, 1993), cardiomyopathy (Obayashi *et al.*, 1992), and ocular myopathy (Fang *et al.*, 1993). In addition to the np 3243 and 3271 MELAS mutations, four other $tRNA^{Leu(UUR)}$ base substitutions result in human disease. A T-to-C transition at np 3250 (Goto *et al.*, 1992) and an A-to-G transition at np 3302 (Bindoff *et al.*, 1993; Shoffner *et al.*, 1993) cause mitochondrial myopathy, a C-to-T transition at np 3303 causes early-onset hypertrophic cardiomyopathy and myopathy (Silvestri *et al.*, 1993), and an A-to-G transition at np 3260 causes late-onset hypertrophic cardiomyopathy and myopathy (Zeviani *et al.*, 1991). This clinical heterogeneity associated with allelic heterogeneity suggest that the individual tRNA nucleotides may vary in function.

This has been suggested for the np 3243 mutation, where both a defect in mitochondrial protein synthesis and in a transcriptional terminator have been proposed. The observed reduction in mitochondrial Complex I (and to a lesser extent, Complex IV) (Kobayashi *et al.*, 1987; Obermaier-Kusser *et al.*, 1991; Ichiki *et al.*, 1988) can be attributed to a defect in mitochondrial protein synthesis detected in skeletal muscle (Moraes *et al.*, 1992a) and in cybrids derived from 3243-positive MELAS patients. A defect in the mitochondrial transcriptional terminator might also be important since the np 3243, but not np 3271, mutation occurs within this sequence (np 3237–3249). This terminator sequence interacts with a *trans*-acting nuclear gene product (m-TERF) to maintain proper rRNA-to-mRNA ratios (Christianson and Clayton, 1986). While alteration of the terminator sequence may be relevant to MELAS, several reports have not been able to substantiate the predicted biochemical changes and, hence, the role of the np 3243 mutation in disrupting transcriptional terminator remains unclear (Suomolainen *et al.*, 1993; Chomyn *et al.*, 1992; Hess *et al.*, 1991; Moraes *et al.*, 1992). However, if both protein synthesis and the rRNA:mRNA ratios are impaired by the 3243 mutation, it suggests an explanation for the range of clinical symptoms associated with the np 3243

mutation which have not been observed for the np 3271 mutation.

A $tRNA^{Leu(UUR)}$ mutation at np 3260 results in MMC, a maternally inherited mitochondrial myopathy featuring hypertrophic cardiomyopathy (Zeviani *et al.*, 1991). This A-to-G transition in the anticodon stem is heteroplasmic, destabilizes a conserved tRNA base pairing, and has not been found in controls. Muscle complex I and IV defects have been attributed to this mutation and the severity of the biochemical defect has been shown to be proportional to the percentage of mutant mtDNAs in skeletal muscle. Since the np 3260 mutation does not disrupt the transcriptional terminator sequence, this mutation probably causes disease by inhibiting mitochondrial protein synthesis.

Overall, the mtDNA tRNA mutations affect both CNS and skeletal/cardiac muscle tissue, while missense mutations primarily affect nervous tissue. For example, among the tRNA mutations, cardiomyopathy results from the $tRNA^{Leu(UUR)}$ np 3260 and 3303 mutations, the $tRNA^{Ile}$ np 4317 mutation (Tanaka *et al.*, 1990), the $tRNA^{Gly}$ np 9997 (Merante *et al.*, 1993) mutation, and occasionally the $tRNA^{Leu(UUR)}$ np 3243 (MELAS) mutation (Obayashi *et al.*, 1992). It is still unclear why the more severe missense mutations do not also cause muscle pathology.

*rRNA Mutations*

Only one mitochondrial rRNA point mutation has been identified which results in human disease. A homoplasmic A-to-G transition at np 1555 of the 12S rRNA gene has been shown to be associated with maternally inherited deafness in one pedigree and maternally inherited aminoglycoside-induced deafness in other cases (Prezant *et al.*, 1993). This mutation has not been found in nearly 300 controls and alters a nucleotide in a highly conserved region of the 12S rRNA gene. In prokaryotic ribosomes this region of the small subunit rRNA constitutes part of the aminoacyl site and is known to interact directly with aminoglycosides, which inhibit bacterial translation. In the aminoglycoside-induced deafness pedigrees, the np 1555 mutation apparently increases the affinity of the antibiotic binding to the mitochondrial 12S rRNA which results in destabilized mitochondrial translation, although no defects in mitochondrial translation products or OXPHOS enzyme activities have been reported (Prezant *et al.*, 1992).

*Interactive Mutations*

The LHON paradigm demonstrates that it is possible for mildly deleterious mtDNA point mutations to accumulate on a mitochondrial chromosome during evolution, thus creating a "predisposing" mtDNA genotype. Since such mutations are not strongly selected against, they do not exhibit a number of the genetic characteristics of the severe mtDNA point mutations, such as heteroplasmy, absence from the unaffected population, and disruption of a highly evolutionarily constrained nucleotide or amino acid. Also, because such mutations are relatively mild, these mutations require additional factors such as the age-related decline in OXPHOS capacity to reduce energy output sufficiently to cause organ failures. Since many years are required to reduce OXPHOS sufficiently to cause disease, these mutations are generally associated with late-onset disease.

Certain mild mtDNA mutations may predispose individuals to Alzheimer's and Parkinson's (AD + PD) disease. AD + PD patients have neuropathological indices of both Alzheimer's (senile plaques and tangles) and Parkinson's (Lewy body formation) disease. In a survey of the mtDNAs of AD + PD patients, an A-to-G transition at np 4336 of the $tRNA^{Gln}$ gene was found at an increased frequency in patients. This homoplasmic mutation alters a moderately conserved nucleotide between the TΨC and aminoacyl acceptor stems of the tRNA. This variant was found in 5.2% (9/173) of Caucasian AD (2/62), PD (2/38), and AD + PD (5/73) patients, but was present in only 0.7% (12/1691) of a random selection of Caucasians. Since these Caucasian "controls" were not age-matched, it is likely that some are actually at risk for developing the disease.

Individuals harboring the rare np 4336 mutation have similar mtDNA haplotypes, such that patients with this mutation cluster in the Caucasian mtDNA phylogeny (Shoffner *et al.*, 1993) (Fig. 3). Both RFLP and mtDNA sequence analysis have revealed the presence of other possibly pathogenic mtDNA mutations in np 4336-positive patients (Shoffner *et al.*, 1993, Brown *et al.*, 1994). One np 4336-positive patient also harbors a missense mutation at np 3397 of the ND1 gene. This homoplasmic variant changes a highly conserved methionine to a valine at the codon adjacent to the LHON np 3394 mutation that converts a tyrosine to a histidine (Brown *et al.*, 1992b). The np 3397 missense mutation was found


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in two independent AD + PD patients, one with the np 4336 mutation and one without. It was not found in 248 Caucasian controls, but has been found in low-frequency in Asia-derived mtDNAs (Shoffner *et al.*, 1993). A second np 4336-positive patient harbored a novel insertion between nucleotides 956–965 in the 12S rRNA gene. This insertion has never been seen before in human mtDNAs ($n$ = roughly 700) and is the first example of an intragenic insertion. A third np 4336-positive patient harbored a T-to-C transition at np 721 in the 12S rRNA gene. This homoplasmic mutation alters a non-conserved nucleotide but has not been found in 905 unaffected individuals including 106 Caucasians (Brown *et al.*, 1994). Thus, the np 4336 mutation may be an important risk factor which predisposes individuals to AD and PD, and the np 3397, 12S rRNA insertion or np 721 mutations accentuate the defect, thus increasing the probability of disease.

## MtDNA REARRANGEMENTS

Pathogenic mtDNA deletions, partial duplications, and arrays of complex rearrangements have also been found in the tissues of patients suffering from OXPHOS disease. Like the deleterious nucleotide substitutions, such rearrangements can result in a broad spectrum of clinical symptoms that can increase in severity and number with age, affect highly oxidative tissues, and are heteroplasmic. Rearrangements can be either spontaneous, nontransmittable mutagenic events or they can be maternally inherited. Genetic differences between the inherited and noninherited rearrangements are still being clarified.

### Spontaneous Deletions

Large-scale mtDNA deletions account for most cases of ocular myopathy and Pearson marrow/pancreas syndrome (Wallace, 1992). Ocular myopathy patients can exhibit a variety of clinical symptoms, ranging from the relatively mild chronic progressive external ophthalmoplegia (CPEO) including ptosis and mitochondrial myopathy to the relatively severe Kearns–Sayre Syndrome (KSS) which features an early age of onset, ophthalmoplegia, atypical retinitis pigmentosa, mitochondrial myopathy, and usually one or more of the following: cerebellar syndrome, cardiac conduction abnormalities, or elevated CSF protein. Pearson marrow/pancreas syndrome patients characteristically exhibit pancytopenia and pancreatic dysfunction. Children surviving early stages of this devastating disease often progress to KSS (McShane *et al.*, 1991).

Greater than 120 different mtDNA deletions have been identified from patient tissues (Wallace *et al.*, 1993b). The mapping and characterization of these deletions indicate common genetic and pathogenic features. First, because ocular myopathy patients are typically sporadic and do not transmit their rearrangements, these deletions must occur either in the oocyte or early in development. Second, the clinical course of patients with mtDNA deletions progresses with age. This phenomenon is probably due to the preferential replication of the smaller deleted molecules resulting in a progressive increase in the mutant/normal mtDNA ratios with age (Larsson *et al.*, 1990). Third, despite extensive heterogeneity in deletion size and breakpoint positions, the two origins of mtDNA replication are spared (Fig. 1). This limits spontaneous deletions to two arcs of the mtDNA chromosome delineated by the two origins, although greater than 90% of mtDNA deletions occur within the larger arc which encompasses 2/3 of the genome. Fourth, deletions occur primarily via a slipped replication mechanism based on mispairing of directly repeated sequences (Shoffner *et al.*, 1989). Direct repeats are common in human mtDNA, in part due to the disproportionate number of guanine residues on the heavy (H)-strand of the mtDNA. Sequence analysis of over 50 pathogenic mtDNA deletions reveals that most occurred within direct repeats of 3–13 nucleotides in length (Wallace *et al.*, 1993b). The most common large-scale mtDNA deletion found in patients is associated with a 13 base pair (bp) direct repeat from np 8470 to 8482 in the ATPase 8 gene and from np 13447–13459 in the ND5 gene (Moraes *et al.*, 1989). The resulting 4997 bp deletion has occurred independently over 100 times and accounts for perhaps 50% of ocular myopathy patients (Wallace *et al.*, 1993b). A second relatively common deletion of 7.4 kb is associated with a 12 bp direct repeat from np 8637 to 8648 in the ATPase6 gene and from np 16073 to 16085 in the noncoding

**Fig. 3.** Phylogenetic tree generated by parsimony analysis from mtDNA RFLP data indicating np 4336-positive lineage associated with a high incidence of AD and PD. Seventy-one Caucasian AD, AD + PD, and PD patients and 74 Caucasian unaffected individuals (c) are shown. Note the independent occurrence of the np 721, 3397, and 12S rRNA insertion (ins) with the np 4336 $tRNA^{Gln}$ mutation in patients.

D-loop (Ozawa *et al.*, 1990; Wallace *et al.*, 1993) (Fig. 1).

Variability in tissue distribution and heteroplasmic ratios in affected tissues can explain much of the clinical heterogeneity observed in patients with spontaneous mtDNA deletions. Typically a patient will harbor a single deletion which is found in the highest mutant/normal ratio in the highly oxidative, postmitotic tissues such as skeletal muscle. The systemic tissue distribution and lack of heritability support the hypothesis that deletions occur either in the egg or early in fetal development. If so, the timing of the deletion event coupled with subsequent mitotic replicative segregation will define the degree of heteroplasmy in different tissues and the tissue distribution of symptoms. Accordingly, in more severe diseases such as Pearson's syndrome or KSS, patients typically have a higher proportion of deleted mtDNAs in more tissues than do CPEO patients.

Data obtained from *in vitro* transcription experiments, *in situ* hybridization studies, and Northern blot analyses of cybrid mtRNAs have shown that both normal and deleted mtDNA molecules are transcribed (Mita *et al.*, 1989, Nakase *et al.*, 1990, Hayashi *et al.*, 1991; Moraes *et al.*, 1992b). Mitochondrial mRNA translation, however, has been shown by immunochemical studies and analysis of $^{35}$S-Met mtDNA translation products to be severely impaired in cells harboring a high proportion of mtDNA deletion (Hayashi *et al.*, 1991; Moraes *et al.*, 1992b). Because virtually all deletions eliminate at least one tRNA, it is likely that deletions result in a generalized translational defect. This would explain why, despite considerable patient-to-patient difference in deletion size and position on the chromosome, ocular myopathy is a common phenotypic result.

## mtDNA Duplications

Partial duplications of the mtDNA have been detected in the mtDNA of ocular myopathy and Pearson's syndrome patients, although duplications are much rarer in these patients than are spontaneous deletions. Duplications are invariably heteroplasmic, can be spontaneous (Poulton *et al.*, 1991) or maternally transmitted (Rotig *et al.*, 1992), and, for the most part, result in clinical phenotypes indistinguishable from those resulting from deletions. In some, if not all cases, duplications may be a component of complex, familial mtDNA rearrangements (see below). In one pedigree, a 26.5 kb chromosome was transmitted from a mother to her two daughters (Rotig *et al.*, 1992). As is the case for deletions, the severity of clinical phenotype is correlated with the proportion of rearranged molecules, as the mildly affected mother harbored low levels of duplicated molecules and exhibited a mild ocular myopathy, while her two daughters inherited higher amounts of the partial duplication and suffered from progressive mitochondrial myopathy, diabetes mellitus, proximal tubulopathy, and ataxia.

Exactly how partial mtDNA duplications arise is unknown (Rotig *et al.*, 1992; Poulton *et al.*, 1993). It has also not been demonstrated why duplicated genes should impair OXPHOS function, although it is easily conceivable that either chimeric gene products which interfere with normal organelle biogenesis or a relative overabundance of duplicated gene products could impede normal mitochondrial function and thus cause human disease.

## Complex mtDNA Rearrangements

Recently, three studies have indicated that complex mtDNA rearrangements can exist within patient tissues. Ballinger *et al.* (1992, unpublished data) have shown that patients belonging to a large maternally inherited pedigree featuring diabetes mellitus and deafness can harbor at least three mtDNA species simultaneously. Extensive Southern blot analysis indicated the presence of partially duplicated (23 kb), normal (16.5 kb), and deleted mtDNA (present as 12 kb dimers) in proband blood and muscle. The partially duplicated and the deleted molecules share the same breakpoint and thus are likely derivatives of the same mutagenic event. Quantitation of the levels of the three species in patients' blood and skeletal muscle consistently showed that the deletion predominated in muscle while the duplication predominated in blood. Further, in lymphoblastoid cell lines established from three patients, the deletion always segregated and was lost while the percentage of duplicated molecules increased. The prevalence of the duplicated molecule in blood and transformed lymphocytes suggests that this molecule can be maternally inherited along with normal chromosomes. If true, two mechanisms could explain the deletion accumulation in muscle: (1) duplicated molecules convert to deleted molecules in skeletal muscle, or (2) deleted molecules are capable of autonomous replication as they contain two

H-strand origins in opposite orientations. Although the proband in this family exhibited severe deficiencies in OXPHOS enzyme activities and mitochondrial translation, the relative contribution of each rearrangement to the pathology remains unclear.

Poulton *et al.* (1993) have reported three patients with KSS plus either diabetes mellitus (two patients) or hypoparathyroidism (the third patient). In each patient, four types of mtDNA molecules were detected: normal, a 24 kb partial duplication (where the duplication was from np 15056 to np 6130, 7.6 kb), a 15.2 deletion dimer, and a 7.1 kb deletion monomer. As in the diabetes and deafness family, the partial duplication was easily detected in blood, and the deleted molecules were prominent in skeletal muscle. When patients were followed longitudinally over a period of 10 years, levels of the partial duplication decreased in skeletal muscle from 16% to 0% (but were maintained in blood), and the level of deletion dimers increased from 19% to 41%. Again, the prevalence and accumulation of deletions in muscle tissue could be due to the conversion of duplicated to deleted form via an unknown mechanism or to a replicative advantage enjoyed by the dimerized deletion which is smaller than wild-type mtDNAs and contains two sets of replication origins. It is unclear what the relative contribution of each rearrangement is to the pathology. Transcription occurs across the breakpoint regions in these patients, suggesting that, if the chimeric RNAs are translated, they interfere in mitochondrial biogenesis.

In each of the above two samples, it is possible that duplicated or partially duplicated mtDNA molecules form a highly "recombinogenic" molecule that essentially predisposes a chromosome to further mutagenic events by either recombination or slipped replication. Moreover, certain mtDNA point mutations may predispose mtDNAs to specific recombination events. A maternally inherited, heteroplasmic, 270 bp tandem duplication has recently been described (Brockington *et al.*, 1993) at low levels in the D-loop of mitochondrial myopathy patients. The duplicated mtDNA contains most of CSB-II, all of CSB-III, and the promoters for both heavy and light strand transcription, the latter of which initiates mtDNA replication, and thus represents a redundancy of important replication and transcriptional control sequences. Further analysis (Torroni *et al.*, 1994) has revealed that a specific Caucasian lineage is prone to this 270 bp duplication because of a mtDNA sequence mutation which creates a region of homology surrounding the duplicated region (Torroni *et al.*, 1994; Torroni and Wallace, this issue). Presumably, this duplication increases the probability of slipped replication and hence the mtDNA rearrangement. This example supports the concept that certain mtDNAs harbor premutations which make them prone to rearrangement. Substantially more studies will be required to clarify the origin, inheritance, propagation, and disease contribution of the various mtDNA rearrangements.

## NUCLEAR GENES AND OXPHOS DISEASE

Nuclear genes encode the majority of polypeptides comprising the electron transport chain and the ATP synthase. At least 33 Complex I, all four Complex II, 10 Complex III, 10 Complex IV, and 11 Complex V proteins are nuclear gene products. Many nuclear proteins are also required for mtDNA replication, transcription, and translation, as well as for nuclear and cytoplasmic genetic regulation. Thus, many aspects of mitochondrial disease must be related to the structure, variation, and regulation of nuclear OXPHOS genes.

Certain mitochondrial diseases must involve faulty interaction of nuclear and cytoplasmic genes. This has been demonstrated in some families with autosomal dominantly inherited mitochondrial myopathy. Affected family members have been found to harbor multiple mtDNA deletions, indicating that a nuclear mutation predisposes these individuals to mtDNA rearrangement (Zeviani *et al.*, 1989). Similarly, nuclear gene variants have been hypothesized to explain the variable expression of LHON or sensorineural deafness in families with homoplasmic mutations. For LHON, the tissue-specific manifestations, the marked bias toward males being affected, and the incomplete penetrance observed in homoplasmic LHON pedigrees have suggested a two-locus pathogenetic mechanism whereby an X-linked susceptibility allele must be co-inherited with the LHON mtDNA mutation. Initial published reports purported to have mapped the X-linked susceptibility allele (Vilkki *et al.*, 1991), but more recent reports have not confirmed this linkage (Chen *et al.*, 1989; Carvalho *et al.*, 1992; Sweeney *et al.*, 1992). Nevertheless, it remains possible that a nDNA gene influences LHON expression.

Likewise, a two-hit model has also been proposed to explain the tissue-specific disease and variable

expression of sensorineural deafness in an Arab family homoplasmic for np 1555 mtDNA 12S rRNA mutation (Prezant *et al.*, 1993), where deafness is congenital and not correlated with antibiotic use (Jaber *et al.*, 1992). Segregation analysis suggested that appearance of patients in this five-generation pedigree could best be explained by co-inheritance of a homozygous autosomal recessive allele together with the np 1555 mtDNA mutation (Jaber *et al.*, 1992; Bu *et al.*, 1993). While these putative nuclear susceptibility alleles have not been mapped, such two-locus pathogenetic mechanism offer a viable hypothesis to explain the incomplete penetrance in maternally inherited diseases due to homoplasmic mtDNA mutations.

The tissue and organ specificity of mtDNA diseases typified by LHON and sensorineural deafness implies that nuclear OXPHOS gene expression must differ between tissues. This could be accomplished by differences in the level of OXPHOS gene expression and the differential expression of tissue-specific OXPHOS isoform genes. The levels of nuclear OXPHOS gene expression vary dramatically between tissues, with the highest levels of ATP synthase and other OXPHOS gene mRNAs occurring in heart and muscle (Stepien *et al.*, 1992; Neckelmann *et al.*, 1989; Williams *et al.*, 1987). Tissue-specific isoforms have been identified for some nuclear OXPHOS genes as both systemic and muscle-specific isoforms have been detected for cytochrome *c* oxidase subunits VIa, VIIa, and VIII (Lomax and Grossman, 1989) as well as for the adenine nucleotide translocator (ANT) (Stepien *et al.*, 1992). In mammals, ANT has three isoforms, encoded by unlinked genes. ANT1 mRNA is present in highest levels in striated muscle, ANT3 mRNA is ubiquitously expressed, and ANT2 mRNA is present at low levels in all tissues (Stepien *et al.*, 1992). Thus, differences in expression of OXPHOS genes may predispose certain tissues to OXPHOS disease pathology by regulating mitochondrial energy output and possibly by specific mtDNA mutations interacting with particular nDNA-encoded, tissue-specific isoforms.

Certain Mendelian diseases have characteristics of known mtDNA diseases and hence might be candidates for diseases due to mutations in nuclear encoded OXPHOS isoform genes. One such disease is facioscapulohumeral muscular dystrophy (FSHD) which is an autosomal dominant neuromuscular disease featuring facial and shoulder girdle muscle weakness. FSHD has been associated with decreased Complex III activity in fibroblasts (Slipetz *et al.*, 1991) and mitochondrial myopathy (Worsfold *et al.*, 1973). The FSHD locus has recently been mapped to chromosome 4 at 4q35, between markers D4S139 and D4S171 (Wijmenga *et al.*, 1993). Since we had previously mapped ANT1 to chromosome 4, we tested ANT1 as a candidate gene for FSHD. Linkage analysis revealed that ANT1 was closely linked to the disease in FSHD families (Haraguchi *et al.*, 1993), but a single recombinant excluded ANT1 as the FSHD gene. This was confirmed by sequencing the ANT1 genes from FSHD patients (Haraguchi *et al.*, 1993). While ANT1 proved not to be the FSHD gene, OXPHOS genes still make attractive candidate genes for certain nuclear-encoded neuromuscular diseases.

One conspicuous feature of mitochondrial myopathy is the enormous proliferation of abnormal mitochondria. It seems that a molecular genetic mechanism must exist that compensates for OXPHOS deficiency by the coordinate induction of OXPHOS genes. This has been confirmed by analyzing nuclear and mtDNA mRNA levels in skeletal muscle of MERRF and MELAS patients. In both groups of patients, nuclear OXPHOS gene (including ANT1 and the $\beta$-subunit of ATP synthase, ATPsyn$\beta$) and mtDNA OXPHOS gene mRNA levels were greatly increased (Heddi *et al.*, 1993). A similar result was seen for KSS patient muscle, with the mtDNA transcripts produced in proportion to their mtDNA copy number as defined by the deleted region. Since deleted mtDNAs are segmentally compartmentalized along a muscle fiber (Shoubridge *et al.*, 1990), it was postulated that nuclear OXPHOS gene induction is limited to these localized regions of the muscle fiber (Heddi *et al.*, 1993).

Another system associated with chronic energy deficiency is ischemic heart disease due to atherosclerotic occlusion of the coronary artery. This starves the heart muscle of substrates and results in periodic and repeated episodes of oxygen and substrate deprivation followed by reperfusion and the resulting oxygen radical production. Analysis of ischemic heart mtDNAs revealed a dramatic induction of mtDNA deletions, 8–2200 times higher than levels found in age-matched healthy hearts. Presumably this is due to oxygen radical mutagenesis of the mtDNA. Furthermore, the expression of ANT1, ANT3, and ATPsyn$\beta$, along with mtDNA transcripts, was also increased, possibly to compensate for energetic deficiencies (Corral-Debrinski *et al.*,

1991). Thus, nuclear OXPHOS gene expression seems to be increased in skeletal muscle and heart when normal ATP production is impaired.

What can account for such alterations of nuclear OXPHOS gene expression in response to poor mitochondrial energy production? Recent studies on promoter regions of nuclear OXPHOS genes in rodents and mammals indicate complex transcriptional regulation accomplished by several *cis*-acting control sequences (Wallace, 1993). So far, developmental transcription studies in humans have only been done for the muscle-specific promoter regions of the ANT1 and ATPsyn$\beta$ genes. The promoters of both of these genes have a common 13 bp (5′-GGCTCTAAAGAGG-3′) *cis*-acting element, the OXBOX, 450 nucleotides upstream of the transcriptional start sites (Li *et al.*, 1990). The OXBOX is a muscle-specific, positive transcription element involved in coordinating ANT1 and ATPsyn$\beta$ genes. Overlapping the OXBOX in both genes is a second element, the REBOX (5′-GCCCATAT-3′) (Chung *et al.*, 1992). This element binds a ubiquitous protein whose affinity is dependent on the redox state of the surrounding solution. Under highly oxidative conditions the factor binds, at physiological conditions it does not, and under highly reduced conditions it is able to bind to the REBOX element again. This biphase binding can be seen with varying concentrations of dithiothreitol or NADH, and has exactly the features that would be expected for an environmental modulator of OXPHOS. When oxygen is plentiful, the cell preferentially makes energy via OXPHOS and needs to increase OXPHOS gene expression. Under physiologically balanced conditions, OXPHOS and glycolysis should be balanced. In patients with mtDNA mutations, mitochondrial energy would be limiting and NADH would accumulate. To compensate for the energy deficiency, the cell would again need to increase the number of mitochondria, which is what occurs. Thus it seems that both nuclear and mitochondrial OXPHOS genes play a critical role in the origin and pathophysiology of OXPHOS disease.

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