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Mitochondrial DNA Mutations and Pathogenesis

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Approximately three years ago, this journal published a review on the clinical and molecular analysis of mitochondrial encephalomyopathies, with emphasis on defects in mitochondrial DNA (mtDNA). At that time, approximately 30 point mutations associated with a variety of maternally-inherited (or rarely, sporadic) disorders had been described. Since that time, almost twenty new pathogenic mtDNA point mutations have been described, and the pace of discovery of such mutations shows no signs of abating. This accumulating body of data has begun to reveal some patterns that may be relevant to pathogenesis.

KEY WORDS: ATP; cardiopathy; deafness; diabetes; encephalomyopathy; KSS; Leigh; LHON; maternal inheritance; MELAS; MERRF; mitochondrial DNA; muscle; MILS; myopathy; NARP; oxidative phosphorylation; PEO; respiratory chain.

INTRODUCTION

The mitochondrial diseases are a highly diverse group of disorders, whose unifying characteristic is the impairment of one or more aspects of mitochondrial function. Typically, these diseases are caused by genetic errors, either in mitochondrial or in nuclear DNA, but some are caused by environmental factors.

Historically, mitochondrial disorders have been a battleground in the debate between the "splitters," who assert that the relationship between genotype and phenotype is relatively tight, and the "lumpers," who feel that different genotypes can cause the same phenotype (and conversely, that the same genotype can cause different phenotypes). The bewildering array and variety of clinical features, biochemical data, morphological findings, and mtDNA genotypes has provided ammunition both for and against the idea that mitochondrial diseases can be categorized "cleanly." The truth probably lies somewhere in between (Rowland, 1994).

In this review, we will enter the fray by reviewing the maternally-inherited errors (mainly mtDNA point mutations) and sporadic errors (mainly mtDNA rearrangements). Mendelian-inherited errors are discussed in the accompanying articles by M. Zeviani and J.-W. Taanman. We will concentrate on those mutations that are beginning to provide insights into possible mechanisms of pathogenesis as well as into basic mitochondrial biology. In addition, we will discuss a series of trends, questions, paradoxes, and potential future directions for research that have begun to emerge.

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MITOCHONDRIAL GENETICS

The human mitochondrial genome is a 16,569-bp circle of double-stranded DNA (Anderson *et al.*, 1981). It is highly compact, and contains only 37 genes (Fig. 1): 2 genes encode ribosomal RNAs, 22 encode transfer RNAs, and 13 encode polypeptides. All 13 polypeptides are components of the respiratory chain/oxidative phosphorylation system, including 7 subunits of complex I (NADH dehydrogenase-ubiquinone oxidoreductase), 1 subunit of complex III (ubiquinone-cytochrome *c* oxidoreductase), 3 subunits of complex IV (cytochrome *c* oxidase), and 2 subunits of complex V (ATP synthetase). The respiratory complexes also contain nucleus-encoded subunits, which are imported into the organelle from the cytosol and assembled, together with the mtDNA-encoded subunits, into the

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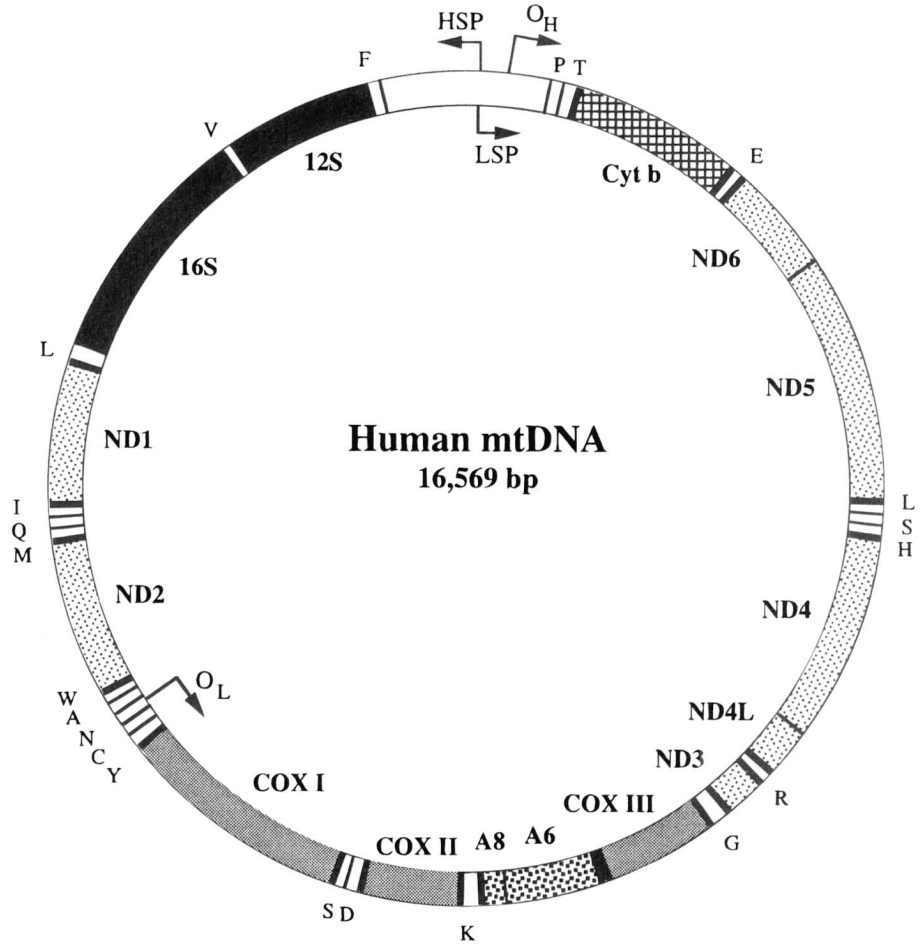

Fig. 1. The human mitochondrial genome. Shown are the structural genes for the 12S and 16S ribosomal RNAs, the subunits of complex I (ND1, ND2, ND3, ND4, ND4L, ND5, and ND6), complex III (Cyt b), complex IV (COX I, COX II, and COX III), and complex V (A6 and A8), and 22 tRNAs (1-letter amino acid nomenclature). The origins of heavy-strand (O_H) and light-strand (O_L) replication, and of the promoters for initiation of transcription from the heavy-strand (HSP) and light-strand (LSP), are shown by arrows.

respective holoenzymes in the mitochondrial inner membrane. Complex II (succinate dehydrogenase-ubiquinone oxidoreductase) contains only nDNA-encoded subunits.

The reader is referred to an earlier review (Schon *et al.*, 1994) for other basic concepts in mitochondrial genetics—maternal inheritance, mitotic segregation, threshold effects, heteroplasmy, and homoplasmy—as they relate to disease, as well as to two databases on the World Wide Web—MITOMAP (http://www.gen.emory.edu/mitomap.html; Kogelnik *et al.*, 1997) and MmtDB (http://www.ba.cnr.it/-area08/mmtdbwww.htm; Calo *et al.*, 1997)—that contain a comprehensive listing of human mtDNA variants.

Because of the central role of oxidative energy metabolism to the viability of the cell, it is not surprising that pathogenic mutations in the mitochondrial genome can have devastating consequences. Moreover, those organs with high oxidative energy requirements, such as brain and muscle, are particularly affected, as relatively few numbers of mutated mtDNAs can affect these tissues adversely. Biochemically, many mitochondrial diseases are characterized by respiratory chain deficiency and lactic acidosis. Morphologically, mitochondria proliferate in tissues of patients; this is seen most strikingly in muscle, where mitochondrial proliferation can be visualized as purplish blotches in the so-called ragged-red fiber (RRF).

DISEASES ASSOCIATED WITH MATERNALLY-INHERITED POINT MUTATIONS IN mtDNA

Most mitochondrial disorders are maternally inherited, and most of these are due to mtDNA point mutations. To date, nearly 50 point mutations have been described (Table I), associated with the following clinical phenotypes:

Leber's Hereditary Optic Neuropathy (LHON)

LHON, a form of blindness due to optic neuropathy, starts in the second or third decade of life and affects men more than women (Leber, 1871). LHON is described in great detail in the accompanying article by E. K. Nikoskelainen, and therefore will not be discussed here.

Myoclonus Epilepsy with Ragged-Red Fibers (MERRF)

MERRF is characterized by myoclonus, generalized seizures, ataxia, and myopathy with ragged-red fibers (Fukuhara *et al.*, 1980). Less consistent clinical features include dementia, short stature, hearing loss, neuropathy, and optic atrophy (Silvestri *et al.*, 1993). Onset is usually in childhood, but adult onset has also been described. Pathological studies have revealed neuronal loss in the inferior olivary nucleus and dentate nucleus, diffuse gliosis of cerebellar and brain stem white matter, and degeneration of the spinal cord posterior columns (Fukuhara *et al.*, 1980). Immunohistochemical studies have shown a selective loss of mtDNA-encoded respiratory chain subunits in frontal cortex, cerebellum, and medulla (Sparaco *et al.*, 1994). Imaging techniques have confirmed the gross pathological findings (Matthews *et al.*, 1993; Huang *et al.*, 1995).

The "classical" MERRF mutation is an A→G transition at nt-8344 in the $tRNA^{Lys}$ gene (Shoffner *et al.*, 1990). Two other MERRF mutations are also located in the $tRNA^{Lys}$ gene, at nt-8356 (Silvestri *et al.*, 1992) and nt-8363 (Ozawa *et al.*, 1997). Four mutations—in $tRNA^{Lys}$ (Zeviani *et al.*, 1993), $tRNA^{Leu(UUR)}$ (Moraes *et al.*, 1993b; Folgero *et al.*, 1995), and $tRNA^{Ser(UCN)}$ (Nakamura *et al.*, 1995)—have been found in patients who had overlap syndromes of MERRF and MELAS (see below). Finally, a syndrome of myoclonus plus deafness was found in a patient with a single-base insertion in $tRNA^{Ser(UCN)}$ (Tiranti *et al.*, 1995). All of these mutations were heteroplasmic.

Other clinical phenotypes have been associated with the nt-8344 mutation, including Leigh syndrome (Silvestri *et al.*, 1993; Howell *et al.*, 1996) and limb-girdle myopathy (Silvestri *et al.*, 1993). An interesting feature of some patients with the nt-8344 mutation is multiple lipomas, be it an isolated manifestation (Holme *et al.*, 1993), as part of the overall MERRF presentation (Berkovic *et al.*, 1991; Silvestri *et al.*, 1993), or as part of a disorder called Ekbom syndrome (Calabresi *et al.*, 1994).

Detailed genetic analyses of the nt-8344 and nt-8356 mutations in cytoplasmic hybrids, or "cybrids" (this is a novel tissue culture system in which mitochondria and mtDNAs from patients can be transferred to immortalized human cells that are devoid of their own mtDNAs [ρ° cells; King and Attardi, 1989]), show that these mutated tRNAs cause both a severe reduction in mitochondrial protein synthesis and the production of a discrete set of aberrant translation products of unknown functional or pathogenic significance (Chomyn *et al.*, 1991; Enriquez *et al.*, 1995; Masucci *et al.*, 1995; Hao and Moraes, 1996). In addition, the $tRNA^{Lys}$ with the nt-8344 mutation is not aminoacylated well by its cognate lysyl-tRNA synthetase (Enriquez *et al.*, 1995). While this may be the cause of some of the translation defects, the quantitative decrease in aminoacylation is probably insufficient to explain all of the biochemical findings.

Mitochondrial Encephalomyopathy with Lactic Acidosis and Stroke-like Episodes (MELAS)

MELAS is the most common maternally-inherited mitochondrial disease. It is defined by: (1) stroke, typically before age 40; (2) encephalopathy characterized by dementia, seizures, or both; and (3) evidence of mitochondrial dysfunction with ragged-red fibers, lactic acidosis, or both (Hirano *et al.*, 1992). Other frequent clinical features include recurrent vomiting, migraine-like headaches, limb weakness, exercise intolerance, and short stature. MELAS families often have oligosymptomatic and asymptomatic maternal relatives (Ciafaloni *et al.*, 1992). Complex I activity appears to be particularly reduced in MELAS (Kobayashi *et al.*, 1987).

The cause of the strokes, which commonly cause hemianopia and cortical blindness, is not known.

Table I. Phenotypes Associated with Pathogenic mtDNA Point Mutations

Nucleotide	Mutation[a]	Gene location	tRNA[b]	"Usual" phenotype	Reference[c]
1555	A→G	12S rRNA	—	Aminoglycoside-induced deafness	Prezant *et al.*, 1993
1642	G→A	tRNA-Val	43	MELAS	Taylor *et al.*, 1996
3243	A→G	tRNA-Leu(UUR)	13	MELAS/PEO/diabetes/deafness	Goto *et al.*, 1990
3250	T→C	tRNA-Leu(UUR)	20	Myopathy	Goto *et al.*, 1992
3251	A→G	tRNA-Leu(UUR)	21	PEO/myopathy	Sweeney *et al.*, 1993
3252	A→G	tRNA-Leu(UUR)	22	MELAS	Morten *et al.*, 1993
3256	C→T	tRNA-Leu(UUR)	25	Multisystem/PEO	Moraes *et al.*, 1993b
3260	A→G	tRNA-Leu(UUR)	29	Cardiomyopathy/myopathy	Zeviani *et al.*, 1991
3271	T→C	tRNA-Leu(UUR)	39	MELAS	Goto *et al.*, 1991
3271	Delete T	tRNA-Leu(UUR)	39	Encephalopathy	Shoffner *et al.*, 1995
3291	T→C	tRNA-Leu(UUR)	60	MELAS	Goto *et al.*, 1994
3302	A→G	tRNA-Leu(UUR)	71	Myopathy	Bindoff *et al.*, 1993
3303	C→T	tRNA-Leu(UUR)	72	Cardiomyopathy	Silvestri *et al.*, 1994
3460	G→A	ND1	—	LHON[d]	Huoponen *et al.*, 1991
4160	T→C	ND1	—	LHON[d]	Howell *et al.*, 1991
4269	A→G	tRNA-Ile	7	Encephalomyopathy/cardiomyopathy	Taniike *et al.*, 1992
4285	T→C	tRNA-Ile	27	PEO	Silvestri *et al.*, 1996
4295	A→G	tRNA-Ile	37	Hypertrophic cardiomyopathy	Merante *et al.*, 1996
4300	A→G	tRNA-Ile	42	Cardiomyopathy	Casali *et al.*, 1995
4320	C→T	tRNA-Ile	63	Hypertrophic cardiomyopathy	Santorelli *et al.*, 1995
5549	G→A	tRNA-Trp	39	Chorea/encephalomyopathy	Nelson *et al.*, 1995
5703	G→A	tRNA-Asn	27	Myopathy/PEO	Moraes *et al.*, 1993b
5814	T→C	tRNA-Cys	13	Encephalopathy	Manfredi *et al.*, 1996
5877	G→A	tRNA-Tyr	15	Myopathy	Sahashi *et al.*, 1994
7445	A→G	tRNA-Ser(UCN)	73	Deafness	Reid *et al.*, 1994
7471	Insert C	tRNA-Ser(UCN)	48	Deafness/myoclonus	Tiranti *et al.*, 1995
7512	T→C	tRNA-Ser(UCN)	5	MERRF/MELAS	Nakamura *et al.*, 1995
7543	A→G	tRNA-Asp	29	Myoclonus	El-Schahawi *et al.*, 1995
8344	A→G	tRNA-Lys	55	MERRF	Shoffner *et al.*, 1990
8356	T→C	tRNA-Lys	65	MERRF	Silvestri *et al.*, 1992
8363	G→A	tRNA-Lys	72	MERRF/deafness/cardiopathy	Santorelli *et al.*, 1996
8851	T→C	ATPase 6	—	FBSN	De Meirleir *et al.*, 1995
8993	T→G	ATPase 6	—	NARP/MILS	Holt *et al.*, 1990
8993	T→C	ATPase 6	—	NARP/MILS	de Vries *et al.*, 1993
9176	T→C	ATPase 6	—	FBSN	Thyagarajan *et al.*, 1995
9957	T→C	COX III	—	MELAS	Manfredi *et al.*, 1995a
9997	T→C	tRNA-Gly	7	Cardiomyopathy	Merante *et al.*, 1994
11696	A→G	ND4	—	LHON/dystonia	de Vries *et al.*, 1996
11778	G→A	ND4	—	LHON[d]	Wallace *et al.*, 1988
12301	G→A	tRNA-Leu(CUN)	37	Sideroblastic anemia	Gattermann *et al.*, 1996
12315	G→A	tRNA-Leu(CUN)	52	Encephalomyopathy	Fu *et al.*, 1996
12320	A→G	tRNA-Leu(CUN)	57	Myopathy	Weber *et al.*, 1997
14459	G→A	ND6	—	LHON/dystonia	Jun *et al.*, 1994
14484	T→C	ND6	—	LHON[d]	Johns *et al.*, 1992
14709	T→C	tRNA-Glu	37	Encephalomyopathy	Hanna *et al.*, 1995
15915	G→A	tRNA-Thr	30	Encephalomyopathy	Nishino *et al.*, 1996
15923	A→G	tRNA-Thr	38	Fatal infantile resp. def.	Yoon *et al.*, 1991
15990	C→T	tRNA-Pro	36	Myopathy	Moraes *et al.*, 1993a

[a] L-strand sequence.
[b] Position on the standard tRNA "cloverleaf" (see Fig. 3).
[c] First published article.
[d] "Primary" pathogenic LHON mutation; so-called "secondary" mutations are not included.

Counterintuitively, imaging shows that the brain lesions do not conform to the distribution of major cerebral arteries (Matthews *et al.*, 1991). Excessive accumulations of mitochondria have been seen in the walls of small arteries and capillaries in muscle and brain (Ohama *et al.*, 1987; Hasegawa *et al.*, 1991). These small vessel abnormalities—called strongly SDH-reactive blood vessels, or SSVs (the angiopathic analog of RRFs, as it were)—are not found in most other mitochondrial disorders (with the notable exception of MERRF [Hasegawa *et al.*, 1993]). It is, however, unclear whether the strokes are due to "small vessel angiopathy" or are, in fact, "metabolic strokes" independent of any vascular pathology.

Another morphological feature unique to MELAS relates to the histochemical stain for cytochrome *c* oxidase (COX) activity. In most mitochondrial disorders associated with tRNA mutations, COX histochemistry in muscle shows a mosaic pattern: some fibers have normal activity whereas others (usually type I fibers) are deficient. RRFs are essentially completely devoid of COX activity, as are the SSVs in MERRF (Hasegawa *et al.*, 1993). In MELAS, on the other hand, the mosaic pattern is still seen, but there are few, if any, COX-negative fibers. In fact, most RRF are COX-positive, although, given the massive proliferation of mitochondria in these fibers, the intensity of the histochemical reaction is much lower than what would be expected. Similarly, SSVs in MELAS are COX-positive.

MELAS has been associated with at least six different point mutations, four of which are located in the same gene, $tRNA^{Leu(UUR)}$. The most common mutation, found in about 80% of cases, is an A→G transition at nt-3243 in the $tRNA^{Leu(UUR)}$ gene (Goto *et al.*, 1990). The three other $tRNA^{Leu(UUR)}$ mutations are at nt-3252 (Morten *et al.*, 1993), nt-3271 (Goto *et al.*, 1991), and nt-3291 (Goto *et al.*, 1994). One mutation was recently reported in $tRNA^{Val}$ (Taylor *et al.*, 1996). All of these mutations were heteroplasmic. Three other tRNA mutations have been found in syndromes overlapping with MERRF (see above).

The prevalence of mutations in the $tRNA^{Leu(UUR)}$ gene in MELAS has always provoked speculation. It is known that the $tRNA^{Leu(UUR)}$ gene has at least two functions: not only is it required to produce a tRNA for translation, but it is also crucial in regulating the amount of ribosomal RNAs the mitochondrion produces. The rRNA region of the mitochondrial genome is transcribed at a much higher rate than is the rest of the genome, in order to produce the large amounts of 12S and 16S rRNAs required to translate the mRNAs encoding the 13 polypeptides (Montoya *et al.*, 1983). Transcription in this region is modulated by a factor, called mTERF, that binds specifically within the $tRNA^{Leu(UUR)}$ gene, thereby promoting termination of transcription (Christianson and Clayton, 1986; Kruse *et al.*, 1989; Hess *et al.*, 1991). The nt-3243 mutation has been shown *in vitro* to impair binding of mTERF to the $tRNA^{Leu(UUR)}$ gene and to reduce the efficiency of transcription termination at the end of the 16S rRNA gene (Hess *et al.*, 1991; Chomyn *et al.*, 1992). The problem with the hypothesis that termination is part of the pathogenetic mechanism in MELAS is that it has not been supported by the few *in vivo* studies performed to date (Hammans *et al.*, 1992; Moraes *et al.*, 1992).

A point mutation in a *polypeptide*-coding gene, at nt-11084 in ND4, had been associated with MELAS (Lertrit *et al.*, 1992), but this is likely to be a nonpathogenic neutral mutation (Ozawa *et al.*, 1991; Sakuta *et al.*, 1993). However, there is now good evidence for a point mutation in a polypeptide-coding gene in a patient with clinically-defined MELAS, at nt-9957 in COX III (Manfredi *et al.*, 1995a). Interestingly, this patient had very few RRF (and all fibers were COX-positive), but had observable COX-positive SSVs in the muscle biopsy. We recently found yet another heteroplasmic mutation in a polypeptide coding gene—in ND5—in a MELAS patient (unpublished data). The histochemical pattern in this patient's muscle was also typical of "classical" MELAS-3243: RRFs were COX-positive, and SSVs (also COX-positive) were present.

The nt-3243 and nt-3271 mutations have been analyzed in cybrids. The major defects observed have been a reduction in protein synthesis (but no production of any aberrant translation products) and a particularly reduced activity of complex I (King *et al.*, 1992; Koga *et al.*, 1993, 1995; Bentlage and Attardi, 1996; Dunbar *et al.*, 1996). In addition, the level of a partially-processed polycistronic RNA composed of 16S rRNA+$tRNA^{Leu(UUR)}$+ND1 (whose genes are contiguous in the DNA; see Fig. 1) was significantly increased in MELAS cybrids and tissues (King *et al.*, 1992; Kaufmann *et al.*, 1996). Since this transcript, called RNA 19, is also present at lower levels in normal tissues, it is unclear whether this increase has any pathogenic significance. However, one possibility is that RNA 19, which contains 16S rRNA, may be incorporated into ribosomes, thereby interfering with translation (Schon *et al.*, 1992). RNA "intermediates" related to RNA 19 have also been observed with

another pathogenic mutation in $tRNA^{Leu(UUR)}$, at nt-3302 (Bindoff *et al.*, 1993).

MELAS-3243 provides indirect evidence that a tRNA mutation can alter the rate of processing of precursor transcripts, but the cause of this altered rate is unknown. One possibility is that the RNA 19 accumulates normally (even in wild-type cells, but at low levels) because the rate at which this precursor is processed into its three constituent RNAs is partially inhibited by a competing molecule (Masucci and Schon, 1996). A good candidate for such a molecule would be the cognate leucyl-tRNA synthetase. If the essential tertiary structure of $tRNA^{Leu(UUR)}$ were present even on the precursor RNA, the synthetase could bind to this "cloverleaf-like" structure while still on the precursor. Such binding would temporarily prevent excision of the tRNA by specific endonucleases (in particular, a ribonucleoprotein called RNAse P). If mutations in $tRNA^{Leu(UUR)}$ were to alter the kinetics of binding of the tRNA-like structure to the synthetase even slightly, one could easily imagine how the steady-state level of an unprocessed molecule such as RNA 19 could increase.

Maternally-Inherited Progressive External Ophthalmoplegia (PEO)

PEO is characterized by weakness of the extraocular muscles and ptosis; it is often accompanied by limb weakness. PEO has been associated with two different types of mtDNA mutations. About one-half of patients with PEO harbor large-scale deletions of mtDNA (see below), which can arise sporadically (Holt *et al.*, 1989; Moraes *et al.*, 1989) or can be inherited as a mendelian trait (Zeviani *et al.*, 1989, 1990; Servidei *et al.*, 1991; Hirano *et al.*, 1994; Bohlega *et al.*, 1996). The remaining patients inherit PEO as a maternal trait, for example, mutations in $tRNA^{Ile}$ (Silvestri *et al.*, 1996), in $tRNALeu^{(UUR)}$ (Sweeney *et al.*, 1993), and in $tRNA^{Asn}$ (Moraes *et al.*, 1993b). Interestingly, many of these patients harbor the nt-3243 mutation, which is more commonly associated with MELAS (Goto *et al.*, 1990; Johns and Hurko, 1991; Moraes *et al.*, 1993c).

In contrast to muscle biopsies from MELAS-3243 patients, which contain mostly COX-positive RRF, the muscle in PEO patients with the nt-3243 mutation contains numerous COX-*negative* RRF. Single-fiber studies have confirmed that there are significantly higher amounts of the nt-3243 mutation in the COX-negative RRFs in PEO as compared to the COX-positive RRFs in MELAS patients, implying that the pattern of the spatial distribution of mutant mtDNAs at the cellular level leads to clinically distinct phenotypes (Petruzzella *et al.*, 1994).

PEO has also been observed in patients with otherwise typical MELAS (Hirano *et al.*, 1992) and MERRF (Silvestri *et al.*, 1993; Moraes *et al.*, 1993b), and is always seen in the sporadic multisystem disorder called Kearns–Sayre syndrome (see below). Two mutations, one at nt-12308 in the $tRNA^{Leu(CUN)}$ gene and the other at nt-15904 in the $tRNA^{Thr}$ gene, had been considered etiologic of PEO (Lauber *et al.*, 1991), but are probably neutral polymorphisms (van den Ouweland *et al.*, 1992a).

Diabetes

One of the most prominent features of mitochondrial disease is endocrinopathy (e.g., diabetes, hypoparathyroidism, hypogonadism, hypothyroidism, adrenal insufficiency, and short stature due to growth hormone deficiency). Thus, while it was perhaps not surprising to find that mtDNA mutations (including mtDNA rearrangements; see below) are associated with endocrine disorders, it *was* surprising to find that a single point mutation—the nt-3243 mutation typically associated with MELAS—is apparently the major cause of maternally-inherited adult type II, non-insulin-dependent, diabetes mellitus (NIDDM). While diabetes can be one of the multisystemic symptoms in MELAS-3243 syndrome (Onishi *et al.*, 1993), there are nt-3243 pedigrees in which diabetes—usually an earlier-onset form of NIDDM—is essentially the exclusive feature (Reardon *et al.*, 1992; Gerbitz *et al.*, 1993). The amount of mutation in the blood of patients with what might now be called maternally-inherited diabetes mellitus (MIDM) is usually lower than that in typical MELAS patients, but no genetic analyses have yet been performed on pancreatic tissue from any of these patients. It was recently shown, however, that pancreatic beta cells most likely require a functioning respiratory chain in order to secrete insulin, because glucose could not stimulate increased insulin production in a mouse pancreatic beta cell ρ^0 line (Soejima *et al.*, 1996). It is estimated that approximately 1–2% of all NIDDM patients in the population harbor the nt-3243 mutation (Kadowaki *et al.*, 1993).

Deafness

Sensorineural hearing loss (SNHL), either partial or total, has emerged as one of the most frequent

symptoms in mitochondrial disease. Deafness, often in conjunction with diabetes, has been noted in maternally-related individuals of pedigrees with the nt-3243 mutation (van den Ouweland *et al.*, 1992b; Remes *et al.*, 1993; Kadowaki *et al.*, 1993; see also below).

Besides being common in MERRF and MELAS, hearing loss was the major, or even the exclusive, symptom in four patients with maternally-inherited mutations. Two of these are in the $tRNA^{Ser(UCN)}$ gene: one is an A→G transition at nt-7445 (Reid *et al.*, 1994) while the other is a single nucleotide insertion at nt-7471 (Tiranti *et al.*, 1995). The third mutation, at nt-8363 in $tRNA^{Lys}$, was found in a patient with cardiopathy and hearing loss (Santorelli *et al.*, 1996; interestingly, another group found the same mutation in a patient with MERRF [Ozawa *et al.*, 1997]). These mutations also cause RRF and lactic acidosis.

The fourth mutation is qualitatively different from the other three. First, it is in a new location: it is the first known pathogenic mutation in a mitochondrial ribosomal RNA gene—an A→G transition at nt-1555 of the 12S rRNA gene (Prezant *et al.*, 1993; Hutchin *et al.*, 1993). Second, it causes deafness through interaction with a class of drugs, namely aminoglycosides, such as streptomycin, kanamycin, and gentamicin (hence the acronym AID—aminoglycoside-induced deafness). Third, the pathogenetic mechanism appears to be novel, as the mutation, which lies near a stem-loop structure in 12S rRNA required for binding of these drugs, probably interferes with mitochondrial translation (Prezant *et al.*, 1993; Hutchin *et al.*, 1993; Inoue *et al.*, 1996). Fourth, the nt-1555 is not associated with either RRF or lactic acidosis.

The nt-1555 mutation can cause nonsyndromic SNHL in pedigrees where aminoglycosides had not been prescribed (Prezant *et al.*, 1993; Matthijs *et al.*, 1996), but that does not rule out the possibility that aminoglycosides, which can be ingested with foods, could still play a pathogenic role in these families. The mutation had been homoplasmic in all families examined to date, but recently we found a Spanish pedigree in which two affected members were homoplasmic and three others were heteroplasmic (El-Schahawi *et al.*, 1997; see also below).

The Striatal Necrosis Syndromes

Paradoxically, one group of diseases provides some of the strongest support for both "lumpers" and "splitters": this is the group of maternally-inherited clinical syndromes that, for want of a better term, we might call the "striatal necrosis syndromes," as they usually display alterations in the basal ganglia upon cranial MRI.

To date, mtDNA mutations causing these syndromes (all heteroplasmic) have been mapped to a single gene—subunit 6 of ATP synthetase (complex V). ATP synthetase is a lollipop-shaped structure composed of two segments: the F_0 segment (the stick) lies in the mitochondrial inner membrane, and conducts protons from the intermembrane space to the matrix, while the F_1 segment (the candy) converts ADP to ATP, using the proton gradient as a source of energy. The two mtDNA-encoded subunits of complex V, ATPase 6 and ATPase 8, are both components of the F_0 proton channel.

In support of the "splitters," it is noteworthy that four mutations in ATPase 6 were identified in two related disorders, called maternally-inherited Leigh syndrome (MILS) and familial bilateral striatal necrosis (FBSN). Leigh syndrome is a devastating multisystem degenerative disorder with onset usually in the first year of life. It is characterized by developmental delay, hypotonia, psychomotor regression, seizures, brainstem dysfunction, myoclonus, ataxia, optic atrophy, and peripheral neuropathy. The diagnosis is confirmed by the characteristic pathological findings of symmetric necrotic foci in the basal ganglia, brainstem, and thalamus. Lactic acidosis is usually present but ragged-red fibers are absent. MILS is associated with high levels (>90%) of either of two mutations—a T→G transversion at nt-8993 or a T→C transition, also at nt-8993 (Tatuch *et al.*, 1992; de Vries *et al.*, 1993; Santorelli *et al.*, 1993, 1994). FBSN shares the symmetrical MRI abnormalities and many of the symptoms of MILS, but the course is more indolent. As with MILS, lactate in FBSN can be elevated but there are no RRFs in muscle. FBSN is associated with two other mutations in ATPase 6—a T→C transition at nt-8851 (De Meirleir *et al.*, 1995) and a T→C transition at nt-9176 (Thyagarajan *et al.*, 1996). All four mutations were heteroplasmic and abundant in examined patients.

In support of the "lumpers," on the other hand, if either of the two MILS-8993 mutations is present at lower levels (about 70%), a completely different disorder ensues: neuropathy, ataxia, and retinitis pigmentosa (NARP) (Holt *et al.*, 1990). NARP is characterized by developmental delay, retinal pigmentary degeneration, seizures, dementia, ataxia, sensory neuropathy, and proximal neurogenic muscle weakness. In contrast to MILS, there are no remarkable findings on MRI.

It has been postulated that the fundamental defect in these disorders lies in the coupling between proton translocation through F_0 and the conversion of ADP to ATP by F_1 (Tatuch *et al.*, 1992). In fact, analysis of cells containing high levels of the T8993G mutation have shown decreases in ATP synthesis of about 50% (Tatuch and Robinson, 1993; Houstek *et al.*, 1995; Vazquez-Memije *et al.*, 1996). It is unclear whether ATP hydrolysis, which is the reverse reaction catalyzed by complex V, is affected by this mutation: one group found no decrease (Vasquez-Memije *et al.*, 1996) whereas another measured a decrease of about 40% (Tatuch and Robinson, 1993). When placed in cybrids, the T8993G mutation caused a reduction in state III (ADP-stimulated) respiration, again on the order of 50% (Trounce *et al.*, 1994). Finally, an engineered mutation in the analogous position in the *E coli* homologue of human ATPase 6 (i.e., ATPase *a*) abolished detectable ATP synthesis via oxidative phosphorylation (Hartzog and Cain, 1993).

Not only have all striatal necrosis mutations been mapped to ATPase 6, but the converse is also true: there is no pathogenic mutation in ATPase 6 associated with any other disorder. It is true that a T→C transition at nt-9101, converting Ile-292 to Thr, was found in a pedigree with LHON (Lamminen *et al.*, 1995), but it is unclear whether this mutation is pathogenic, as it was homoplasmic in all examined pedigree members and was not in a conserved region of the polypeptide.

Cardiomyopathy

Patients with "classical" MERRF-8344 and MELAS-3243 have signs of cardiac involvement, including cardiomegaly, septal hypertrophy, and ventricular hypertrophy (Anan *et al.*, 1995). However, a new trend has emerged in the last few years, that is, the finding of a separate group of mtDNA point mutations in patients with predominantly, or even exclusively, maternally-inherited cardiomyopathies (MICM). In addition, the type of cardiac involvement with these mutations—mainly hypertrophic cardiomyopathy—is different from the cardiac conduction abnormalities characteristically seen in patients with sporadic Kearns–Sayre syndrome who harbor giant deletions of mtDNA (see below).

A search in 1992 for mtDNA mutations associated with hypertrophic cardiomyopathies had turned up numerous candidate mutations (Obayashi *et al.*, 1992), but the pathological relevance of those mutations was unclear. More recent work, however, has revealed that there indeed are "cardiomyopathy-specific" mutations. It is striking that four of them are located in one gene, $tRNA^{Ile}$ (at nt-4269 [Taniike *et al.*, 1992], nt-4295 [Merante *et al.*, 1996], nt-4300 [Casali *et al.*, 1995], and nt-4320 [Santorelli *et al.*, 1995]). As with the "hotspot" of MELAS mutations in the $tRNA^{Leu(UUR)}$ gene, the reason for the concentration of cardiomyopathy-related mutations in $tRNA^{Ile}$ is unknown. The other cardiomyopathy mutations are also in tRNAs, at nt-3260 and nt-3303 in $tRNA^{Leu(UUR)}$ (Zeviani *et al.*, 1991; Silvestri *et al.*, 1994), and at nt-9997 in $tRNA^{Gly}$ (Merante *et al.*, 1994).

Three other mutations associated with cardiomyopathies deserve special mention. (1) A mutation at nt-8363 in $tRNA^{Lys}$ has been described in a patient with MERRF (Ozawa *et al.*, 1997), but we have found the same mutation in a pedigree whose main clinical features were cardiopathy and hearing loss (Santorelli *et al.*, 1996). (2) Hypertrophic cardiomyopathy was a novel feature in an infant with Leigh syndrome harboring the T8993G mutation in the ATPase 6 gene (Pastores *et al.*, 1994). (3) Finally, and perhaps most unusual, a maternal pedigree was recently found in which members had heteroplasmic levels (ranging from about 60–95%) of the nt-1555 mutation classically associated with AID/SNHL, but amazingly, cardiomyopathy was the only clinical manifestation (F. Santorelli and M. Hirano, personal communication).

Other Disorders

Maternally-inherited myopathies have been associated with mutations at nt-3250, nt-3251, and nt-3302 in $tRNA^{Leu(UUR)}$ (Goto *et al.*, 1992; Sweeney *et al.*, 1993; Bindoff *et al.*, 1993), at nt-5703 in $tRNA^{Asn}$ (Moraes *et al.*, 1993b), and at nt-5877 in $tRNA^{Tyr}$ (Sahashi *et al.*, 1994). Fatal infantile respiratory chain deficiency with multisystem involvement was associated with a mutation at nt-15923 in $tRNA^{Thr}$ (Yoon *et al.*, 1991).

Two mutations have been linked to dystonia (typically a mendelian-inherited disorder). Interestingly, both mutations were found in pedigrees with LHON, and both are in subunits of complex I (ND4 [de Vries *et al.*, 1996] and ND6 [Jun *et al.*, 1994]).

Two recently-identified mutations show that the spectrum of clinical phenotypes has not yet been exhausted. One, in $tRNA^{Trp}$, was associated with an encephalomyopathy in which chorea was a distinguish-

ing sign (Nelson *et al.*, 1995), and the other, in tRNA-$^{Leu(CUN)}$, was associated with sideroblastic anemia (Gatterman *et al.*, 1996). The latter disorder is reminiscent of Pearson's marrow/pancreas syndrome, which is typically associated with spontaneous deletions of mtDNA (see below).

DISEASES ASSOCIATED WITH SPORADIC REARRANGEMENTS OF mtDNA

As opposed to the maternally-inherited point mutations, one group of mtDNA mutations occurs spontaneously, with no obvious genetic component. These mutations fall into a completely different genetic category, namely, the large-scale partial deletions and duplications of mtDNA. Since they were first discovered in 1988 (Holt *et al.*, 1988), almost 200 species of rearranged mtDNAs have been reported.

Kearns–Sayre syndrome (KSS), which was first recognized as a distinct clinical entity almost 40 years ago (Kearns and Sayre, 1958), is the most prominent disorder associated with mtDNA rearrangements. It is defined by the triad of PEO, pigmentary retinopathy, and onset before age 20, with at least one of the following additional features: cerebellar syndrome, cardiac conduction block, or cerebrospinal fluid protein greater than 100 mg/dl (Rowland *et al.*, 1983). Other features of KSS include hearing loss, endocrinopathies (particularly hypoparathyroidism and diabetes mellitus), proximal limb weakness, and renal tubular dysfunction. Blood and CSF have elevated levels of lactate, and muscle biopsies show RRF. The course is relentlessly downhill and death usually occurs at a young age.

In 1988, giant deletions of mtDNA (Δ-mtDNAs) were found in patients with KSS and in patients with isolated ocular myopathy, a less severe sporadic disorder also associated with PEO (Zeviani *et al.*, 1988; Lestienne and Ponsot, 1988; Holt *et al.*, 1989; Moraes *et al.*, 1989). The same Δ-mtDNAs seen in KSS and PEO were also found in an early-onset hematopoetic disorder called Pearson's marrow/pancreas syndrome (Rötig *et al.*, 1991), which is characterized by sideroblastic anemia and exocrine pancreatic dysfunction; the anemia can be fatal despite blood transfusion therapy (Pearson *et al.*, 1979). The few patients who survive Pearson syndrome (PS) later develop KSS (McShane *et al.*, 1991).

In all three of these disorders, the Δ-mtDNAs are observed easily by Southern blot hybridization analysis as a large population (up to 80% of total mtDNA) of a single species of mtDNA migrating in electrophoretic gels more rapidly than do full-length mtDNAs. While the specific type of deletion varies among patients, about one-third of all KSS/PEO/PS patients have the same deletion, called the "common deletion" (Schon *et al.*, 1989; Mita *et al.*, 1990), which removes 4,977 bp of mtDNA between the ATPase 8 and ND5 genes (see Fig. 2).

An unusual *small*-scale deletion has also been described. Keightley *et al.*, (1996) identified a patient with recurrent myoglobinuria and COX deficiency who harbored a heteroplasmic mtDNA microdeletion of only 15 bp located within the COX III gene; the mutation thus removed five in-frame amino acids in the polypeptide. While the mutation was absent from the blood of the patient's clinically normal mother, it could have been maternally inherited, as muscle was not available for analysis.

Duplications of mtDNA (dup-mtDNAs) have also been identified in sporadic KSS (Poulton *et al.*, 1989, 1993, 1995; Brockington *et al.*, 1995) and PS (Rötig *et al.*, 1995), but not as frequently as deletions. The lower frequency of observed dup-mtDNAs may be due, in part, to the difficulty in distinguishing duplicated from deleted species: digestion of mtDNA with restriction enzymes normally used for mtDNA Southern blot analysis generates the identical heteroplasmic pattern of a wild-type fragment of 16.6-kb plus a smaller fragment representing either an authentic partially-deleted molecule or the rearranged portion of an authentic partially-duplicated molecule, or both. Digestion with enzymes that cut only once in dup-mtDNA (and not at all in Δ-mtDNAs), and the use of appropriate regional hybridization probes, can reveal the presence of dup-mtDNAs in samples that had previously been thought to harbor only Δ-mtDNAs (see Fig. 2).

When such an analysis is performed, an important feature emerges: the dup-mtDNAs and Δ-mtDNAs present in any one patient are related structurally: the partially duplicated molecule is essentially a tandem duplication of wt-mtDNA in which the material missing in the analogous Δ-mtDNA has been removed, or viewed alternatively, the duplicated molecule is a wt-mtDNA into which the Δ-mtDNA has been inserted. (e.g., for the "common deletion" of 4,977 bp, the Δ-mtDNA is 11,592 bp long [i.e., 16,569 − 4,977] and the dup-mtDNA is 28,161 bp long [i.e., 16,569 + 16,569 − 4,977, equivalent to 16,569 + 11,592]). The relationship between duplications and deletions is still not clear, but it seems likely that duplications are

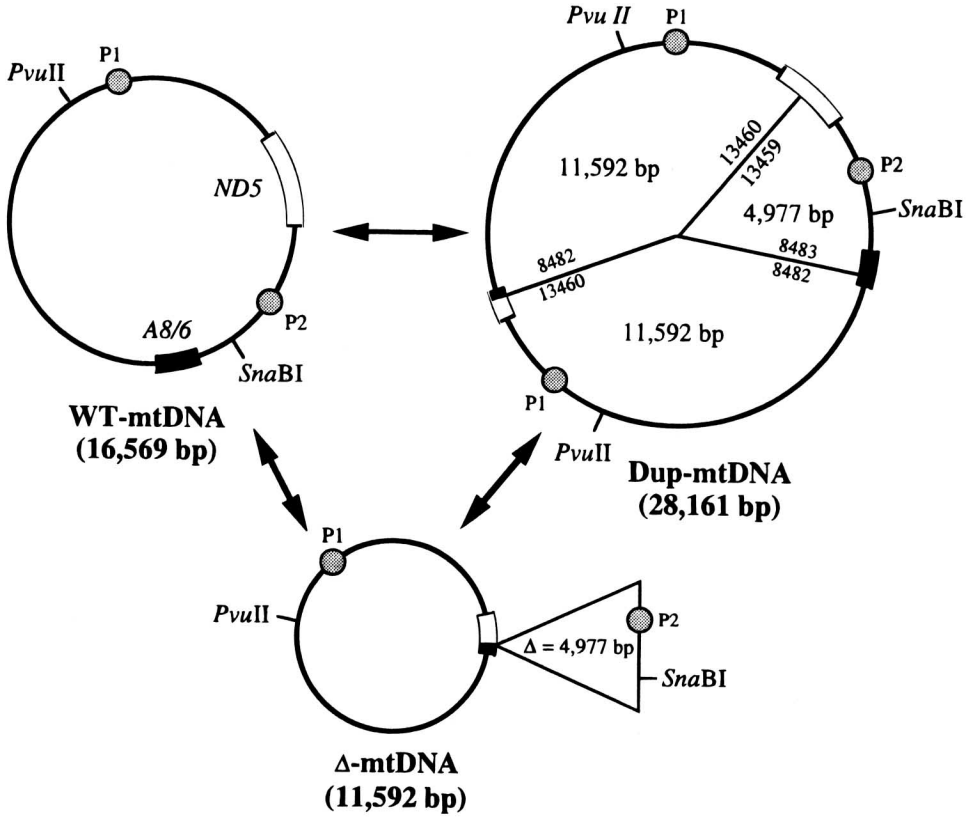

Fig. 2. Rearrangements in human mtDNA. Using the "common deletion" as an example, the proposed relationships among wild-type (WT), deleted (Δ), and duplicated (dup) mtDNAs are shown. Only the genes located at the rearrangement breakpoint are shown, along with the breakpoint map coordinates (at the "pie" section boundaries). *Pvu*II and *Sna*BI are examples of a pair of restriction enzymes that, in conjunction with suitable probes (P1, P2) located inside or outside the deletion, can be used to distinguish among the three species by Southern blot hybridization analysis.

recombination intermediates which can be resolved into deletions (Poulton *et al.*, 1993) (see Fig. 2). Thus, a KSS patient thought to harbor only the common deletion (Shanske *et al.*, 1990) was re-examined using a combination of Southern blot analyses and a new long PCR technique that is able to amplify dup-mtDNAs specifically (Fromenty *et al.*, 1996). This analysis revealed that the patient harbored both types of rearrangements (Fromenty *et al.*, 1997). Thus, it is likely that a sizable number of KSS patients have both deletions and duplications, and perhaps other related rearrangements, such as deletion dimers, as well.

There is only a single type of rearranged molecule in any one patient with sporadic KSS/PEO/PS. This finding implies that the population of rearranged mtDNA molecules in such patients is a clonal expansion of a single spontaneous deletion and/or duplication event occurring early in oögenesis or embryogenesis. In support of this idea, very low levels of mtDNA rearrangements have been found in the oocytes of *normal* women (Chen *et al.*, 1995). Human oocytes contain about 100,000 mtDNAs (Chen *et al.*, 1995), of which approximately 1,000 will eventually repopulate the fetus. If only one rearranged mtDNA somehow "slipped through" this bottleneck and entered the child, it would explain three aspects of these diseases: (1) why they are sporadic; (2) why the rearrangements are "clonal"; and (3) why the diseases are so rare.

Once fertilization occurs, there is no mtDNA replication until the blastocyst stage of development (which is the time when germ layer differentiation begins). It is easy to envision how a few rearranged mtDNAs present at or immediately following the blastocyst stage could segregate in a highly skewed fashion among the germ layers (or even among their progeny cells), in which case one would predict that mutated mtDNAs entering all 3 germ layers would result in KSS (a multisystem disorder), segregation to the hema-

topoetic lineage would result in PS, and segregation to muscle would result in PEO (Schon *et al.*, 1991). Interestingly, two apparently *sporadic* tRNA mutations support the concept of segregation, as both—one in $tRNA^{Pro}$ (Moraes *et al.*, 1993a) and the other in $tRNA^{Leu(CUN)}$ (Weber *et al.*, 1997)—resulted in pure myopathies with no apparent involvement of other tissues, and with no detectable mutation in examined nonmuscle tissues.

The hypothesis of clonality implies that a single rearranged molecule present in vanishingly small amounts at fertilization can give rise to the trillions of Δ-mtDNAs that are detected in a KSS patient and that will eventually kill that patient. How does this selective amplification of Δ-mtDNAs occur? Some investigators believe that the Δ-mtDNAs "take over" the patient because they are smaller, and thereby replicate faster than do wild-type mtDNAs. However, the only relevant experiment reported to date implies that this is not the case (Moraes and Schon, 1995). It seems that there are more subtle signals that determine proliferative advantage for Δ-mtDNAs.

The pathogenicity of mtDNA deletions is well established, most strongly from cybrid experiments (Hayashi *et al.*, 1991), and is probably related to the fact that deletions invariably remove tRNA genes which are required for translation of mtDNA-encoded mRNAs (Nakase *et al.*, 1990). The tRNA hypothesis would also explain why even the *undeleted* genes are not translated (Mita *et al.*, 1989; Davidson *et al.*, 1995), and would also explain why KSS patients with different Δ-mtDNAs display similar biochemical and morphological signs.

On the other hand, it is still unclear whether mtDNA duplications are directly pathogenic. Since tRNA genes are *not* missing in dup-mtDNAs, other pathogenetic mechanisms would have to be invoked. Perhaps the very excess of tRNAs (derived from the duplicated segment) causes a problem, as there would now be an unbalanced amount of tRNAs that might affect tRNA turnover or translation in general. A more likely possibility is that the chimeric fusion gene straddling the abnormal duplication breakpoint is translated and is incorporated into a respiratory chain complex, thereby compromising its function. If the latter hypothesis were true, it would imply that, as opposed to the relatively uniform clinical and biochemical phenotypes seen with Δ-mtDNAs, different dup-mtDNAs could have very different consequences, depending on the type of chimeric gene formed at the duplication breakpoint.

DISEASES ASSOCIATED WITH MATERNALLY-INHERITED REARRANGEMENTS OF mtDNA

While most DNA rearrangements arise spontaneously, there is no theoretical reason why they could not be transmitted as a maternally-inherited trait. In fact, maternally-inherited mtDNA duplications have been observed, with diabetes as the main, or even sole feature (Rötig *et al.*, 1992; Dunbar *et al.*, 1993; Ballinger *et al.*, 1994). As with the $tRNA^{Leu(UUR)}$ mutation, the diabetes is usually the type II, non-insulin-dependent, form, although insulin-dependent diabetes has also been described (Superti-Furga *et al.*, 1993). Interestingly, pedigrees with maternally-inherited diabetes had been described more than twenty years ago (Dorner and Mohnike, 1976).

While there is ample evidence of maternal transmission of mtDNA duplications, there is, surprisingly, almost no solid evidence that large-scale mtDNA deletions can be transmitted. A mother with sporadic PEO and ostensibly deleted mtDNA was reported to have transmitted the mtDNA rearrangement to her child, who was diagnosed with Pearson syndrome (Bernes *et al.*, 1993), but the data do not rule out the possibility that the rearrangement was actually a duplication.

Maternal inheritance of a heteroplasmic, *small*-scale mtDNA rearrangement has been described in one case. Brockington *et al.* (1993) found a sporadic KSS patient who harbored a typical large-scale deletion plus an unrelated tandem duplication of a 260-bp segment of noncoding mtDNA located in the D-loop region. The duplication, but not the deletion, was present in the patient's clinically normal mother, implying that the duplication was not pathogenic. Manfredi *et al.* (1995b) found a similar patient with a sporadic myopathy (not KSS) who also had heteroplasmic levels of this 260-bp rearrangement; analysis in cybrids supports the view that this tandem duplication is not pathogenic (Hao *et al.*, 1997).

PATHOGENESIS

It is clear that the discoveries of the last few years, mainly in molecular biology, have transformed our understanding of mitochondrial diseases. Unfortunately, almost all of our understanding has been confined to etiology. It is no small achievement that we can now predict with some certainty that a specific mtDNA mutation will likely cause mitochondrial dys-

function, and which specific disease (or at worst, what specific range of symptoms) a particular mutation will lead to. Even more remarkable, this knowledge now enables us to screen for specific mtDNA mutations, not only to validate a diagnosis where the clinician suspects a particular syndrome, but also to search for, and often find, candidate etiologic mutations in those cases where the clinical findings are ambiguous.

Pathogenesis, on the other hand, is a largely undiscovered country. On the whole, we do not know why a specific mutation causes a specific disease or constellation of symptoms, and this is true even when we have a fairly good idea as to what specific biochemical error follows from a particular genetic error. For example, an mtDNA deletion results in an inability to translate mitochondrial mRNAs, presumably due to the absence of the tRNA genes that happen to reside within the deletion. Similarly, we know that the MERRF mutation at nt-8344 in $tRNA^{Lys}$ impairs translation severely, and that the MELAS mutation at nt-3243 in $tRNA^{Leu(UUR)}$ also impairs translation, but to a lesser extent. What we don't understand is why the inhibition of translation in these three cases leads to three different clinical phenotypes.

One fact that we do know is that the quantitative aspects of mitochondrial genetics—heteroplasmy, mitotic segregation, and threshold effects—play a crucial role in pathogenesis. For example, individuals carrying the nt-3243 mutation can have at least three diseases: diabetes (if the level of mutation is "low," say 40–50% in blood), PEO (if the level is "intermediate," say 60–70% in muscle), and MELAS (if the level is "high," say 80–95% in muscle or brain). In addition, the concentration of the mutation in cells or groups of cells likely determines phenotype. This would explain the paradox posed by patients with the nt-3243 mutation who have either PEO or MELAS: patients with PEO-3243 have only about 60% mutation in muscle *overall*, yet most RRF in PEO-3243 muscle are COX-*negative* and contain >95% mutation; on the other hand, patients with MELAS-3243 have 80% mutation in muscle *overall*, but most RRF are COX-*positive* and contain <95% mutation. The simplest explanation of the data is that the presentation of two phenotypes associated with the same genotype is determined mainly by the localized concentration and distribution of the mutation in affected tissues (Petruzzella *et al.*, 1994).

Presumably, the lesson of PEO/MELAS-3243 can be generalized to other mutations and other tissues as well. Thus, high levels of Δ-mtDNA in blood but not muscle cause Pearson syndrome but not sporadic PEO; high levels in muscle but not blood causes PEO but not PS; and high levels in all tissues causes KSS. By extrapolation, some deletion-related disorders may still await discovery, based on selective distributions of Δ-mtDNAs in particular tissues that have yet to be identified.

Ever since the first mutations were identified in 1988, the essential question, and one which has crystallized the lumper–splitter debate, has been that of specificity: why are specific mutations associated with specific syndromes? Why is MELAS predominantly associated with mutations in $tRNA^{Leu(UUR)}$ and MERRF with mutations in $tRNA^{Lys}$? If all mutations in mtDNA affect respiratory chain/oxidative phosphorylation, why doesn't every mutation cause pretty much the same syndrome? Paraphrasing Tolstoy, all neutral polymorphisms are similar in their behavior, but each pathogenic mutation appears to be pathogenic in its own way.

Then there is the issue of "symptom-specificity": why does a $tRNA^{Leu(UUR)}$ mutation at nt-3243 cause strokes (but not cardiopathy), whereas a $tRNA^{Ile}$ mutation causes cardiopathy (but not strokes)? Why does 90+% reduction in mitochondrial protein synthesis due to a deletion cause cardiac conduction block, while a similar reduction due to a tRNA mutation does not?

One of the few clues towards solving the specificity problem is an intriguing observation regarding point mutations in tRNAs. There are a few cases in which essentially the same clinical presentation is associated with different mutations in different tRNA genes, but the mutation is at the *same position* on the tRNA itself. For example, mutations at nt-4269 in $tRNA^{Ile}$ and at nt-9997 in $tRNA^{Gly}$ both cause maternally-inherited cardiomyopathy. It turns out that both mutations map to the identical position on the canonical 73-nucleotide tRNA "cloverleaf" structure (Sprinzl *et al.*, 1989), at nucleotide position #7, in the acceptor stem of the molecule (Fig. 3). Similarly, two other cardiomyopathy mutations, at nt-3303 in $tRNA^{Leu(UUR)}$ and nt-8363 in $tRNA^{Lys}$, map to tRNA position #72, also in the acceptor stem. Thus, it may be that phenotype and genotype *are* related, not at the DNA sequence level but at the level of secondary or tertiary structure. This implies that a particular alteration in generic tRNA structure has a specific consequence that we perceive as a specific clinical disorder (if true, this outcome would be a satisfying way to reconcile the lumpers and the splitters). Efforts to study the biophysics of

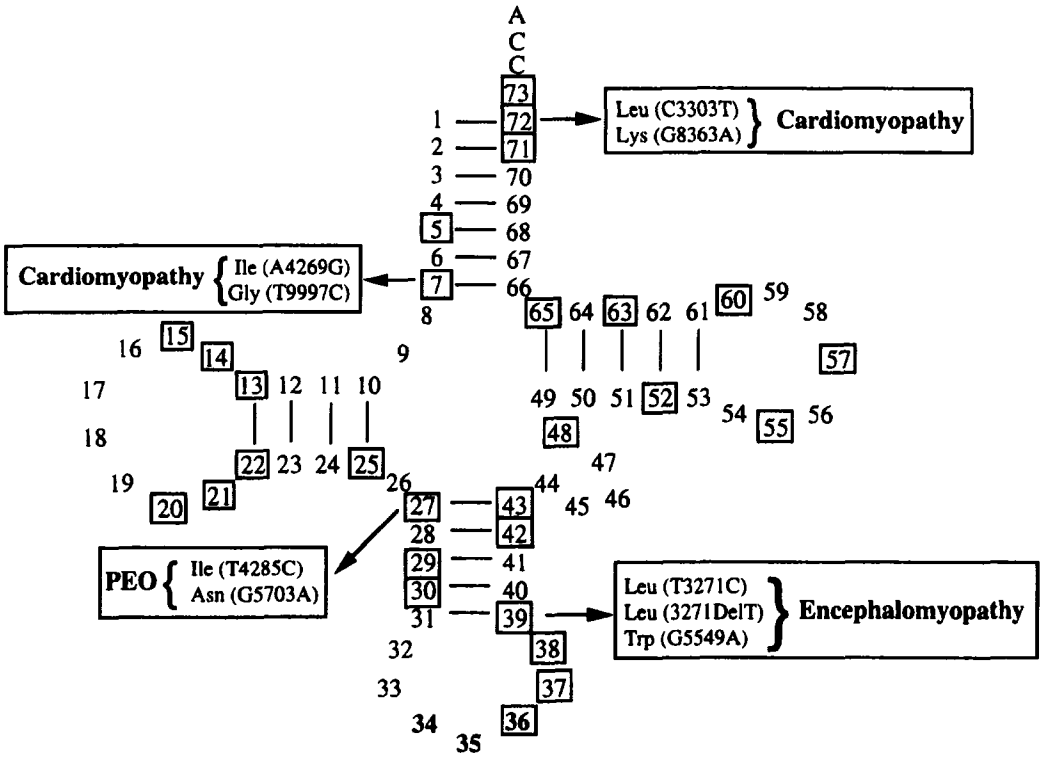

Fig. 3. The generic tRNA "cloverleaf," with standard nucleotide numbering (Sprinzl *et al.*, 1989). Positions of pathogenic mitochondrial tRNA point mutations (see Table I) are boxed. Four sets of mutations are highlighted, in which mutations at the same tRNA position, irrespective of the particular tRNA in which the mutation is located, are associated with essentially the same clinical presentation.

these mutations might thus be a fruitful line of investigation.

The mutations associated with LHON challenge almost every aspect of our thinking about the biology of mitochondrial disease. These can be summarized in a series of questions. Why are all known LHON mutations in polypeptide-coding genes? Since many patients harbor homoplasmic or nearly-homoplasmic levels of the mutation in all of their tissues, why are only eye and optic nerve typically affected the most severely (or even exclusively)? Why is the respiratory deficiency so mild? Why is the onset of the disease relatively late compared to other homoplasmic mtDNA mutations? When patients become blind, why are both eyes affected essentially simultaneously (within a period of a few weeks or months)? In those rare instances where it occurs, why is the blindness reversible? Why are men far more susceptible to LHON than are women? In essence, within the context of the other disorders described in this review, it is difficult to call LHON a mitochondrial disease at all, aside from the obvious fact that it is associated with mtDNA mutations. The possibility that LHON might be an autoimmune disorder did not escape the attention of the late Anita Harding (Harding *et al.*, 1992; Kellar-Wood *et al.*, 1994).

The biochemical analyses of the T8993G mutation found in NARP and MILS pose a particularly interesting problem. As noted earlier, fibroblasts and lymphocytes harboring >90% mutation show a decline in ATP synthesis of approximately 50%. If these analyses *in vitro* actually reflect what takes place in neurons *in vivo*, we are left with an amazing conclusion: that chronic depletion of the steady-state levels of a cell's ATP by *only half* is sufficient to kill that cell, and the patient. One might expect cell death to occur at a 90% decrease of ATP synthesis, but a lethal 50% decrease is harder to believe, as this level is probably achieved often during a cell's normal range of activities. There are many possible answers to this question. It may be that *in vivo* the degree of reduction in ATP synthesis is, in fact, closer to 90% than to 50%; it may be that ATP synthesis in neurons is limited by substrate availability (Vasquez-Memije *et al.* [1996] found a

50% reduction in ATP synthesis using succinate as a substrate, but a 90% reduction using malate); it may be that a *chronic* reduction of ATP to 50% is lethal, but that acute reductions during normal cellular activities are not.

Thus, in spite of our greater appreciation and understanding of the role of population genetics in mitochondrial disease, the essential pathogenetic mechanisms, and the fundamental reasons for tissue- and symptom-specificity, remain elusive. Perhaps we are looking for clues in the wrong places. One clue that seems of particular relevance, in our view, is related to the organ systems that are on the whole affected, *and unaffected*, in mitochondrial disease. The current, and to a great extent probably the correct, view, is that mitochondrial diseases affect long-lived postmitotic tissues with high oxidative energy requirements. This would explain why most mitochondrial disorders affect muscle, heart, brain, eye, and endocrine tissue, while there are very few reported mitochondrial disorders involving liver (except for severe mtDNA depletion [Moraes *et al.*, 1991] see also the article by M. Zeviani), skin, bile duct, lung, bone, or connective tissue.

There is another way of viewing the difference between affected and unaffected organ systems, however. To a first approximation, affected organs are involved in extracellular transport of small molecules and ions across the plasma membrane (most notably, but not exclusively, electrically excitable tissues, such as muscle and brain, which exchange Na^+ and K^+), whereas relatively unaffected organs are not. This division also extends to the defining symptoms that are the hallmarks of particular mitochondrial diseases: heart conduction block in the case of KSS; paralysis of extraocular muscle in the case of PEO; myoclonus in the case of MERRF; neuronal dysfunction in the striatal necrosis syndromes; defects in sensorineural tissue in the case of deafness.

Transporter defects would also explain why a tissue like kidney is frequently affected (Superti-Furga *et al.*, 1993; Rötig *et al.*, 1992; Naiudet *et al.*, 1994; Szabolcs *et al.*, 1994; Hinokio *et al.*, 1995). In addition, it is thought that the main function of astrocytes is to regulate the ionic milieu between neurons and the vasculature. If transport in astrocytes were compromised by mtDNA mutations, spongiform degeneration in both white and gray matter would be expected to occur. In fact, such degeneration (which seems to be caused by brain edema) is the main neuropathologic feature of mitochondrial encephalopathies, especially KSS and Leigh syndrome.

For other "mitochondrion-selective" symptoms, the involvement of "transporter" tissue can also be envisioned. These include the putative involvement of the glucose sensor/transporter in the case of diabetes, of vascular smooth muscle in the case of the strokes in MELAS, and of the retinal pigment epithelium (RPE) in the case of the pigmentary retinopathy found in KSS.

On this latter point, we note that the RPE is part of the blood/eye barrier, and one of its main tasks is to nourish the retina with appropriate solutes while preventing the passage of undesirable ones. The RPE has a functional analog in the brain, the choroid plexus (CP). The CP generates the cerebrospinal fluid while at the same time preventing the passage of undesired molecules across the blood/brain barrier. In fact, the CP is not only functionally, but also anatomically, equivalent to the RPE, as the two organs arise from the same precursor tissue (which explains why the primordium of the RPE in the developing eye has been called the "fifth ventricle" of the brain). Thus, it may well be that two ostensibly unrelated symptoms in KSS—pigmentary retinopathy and elevated CSF protein—are in fact highly related, in the sense that both features may be due to the inability of these barrier tissues to regulate solute/solvent transport effectively. One would also predict that both the RPE and the CP in any one KSS patient would contain essentially the same [high] amount of mutated Δ-mtDNA, and that the pattern of "oligoclonal" bands in a KSS patient's CSF would be essentially similar to the pattern in blood (because the elevated CSF protein is due to inappropriate leakage of polypeptides from the blood into the CSF through the "leaky" CP). In truth, there are only two reports of mitochondrial pathology in the choroid plexus, one in a patient with MELAS (Ohama *et al.*, 1987) and the other in a woman with Leigh syndrome (Kepes, 1983), but the CP is not a tissue that is routinely investigated in mitochondrial disease.

As noted above, the "machines" that are most closely associated with transport are usually located in the plasma membrane, specifically, channels and pumps of various kinds. These include ion pumps for sodium, potassium, and calcium, and transporters for molecules, such as lactate, glucose, and neurotransmitters. Unsurprisingly, many (perhaps most) of these channels, transporters, and pumps are dependent on ATP for their functioning (i.e., they are ATPases). Perhaps when the amount of ATP in the cytosol falls below a certain level, these ATP-dependent proteins

are particularly affected, thus leading to the symptoms most prominently seen in mitochondrial disease.

One argument against this hypothesis is that the cytosolic ATP derived from glycolysis ought to be able to run these pumps even when ATP derived from mitochondrial oxidative phosphorylation is reduced. We think, however, that glycolytic ATP cannot substitute for mitochondrion-derived oxidative ATP to perform these tasks, for two reasons: too little of it is present and too little of it is *available*. So much ATP is required at the plasma membrane to run pumps and channels that ATP is probably diffusion-limited, and glycolysis could never provide enough ATP molecules at the site of usage to be an efficient alternative source of energy for these pumps. On the other hand, it is not hard to envision mitochondria as devices for carrying ATP directly to the point of usage, located adjacent to these transporters.

An extreme, but useful, example of this concept of "ATP compartmentation" is the neuron. Ion pumping requires the most ATP in brain, and almost all of the supply of energy comes from the metabolism of glucose (Erecinska and and Silver, 1989). Nevertheless, electrical transmission (i.e., Na^+-K^+ exchange across the plasma membrane) occurs in elongated regions of the neuron with low volume (i.e., relatively low amounts of cytoplasm) located far away from the cell body. Similarly, neurotransmitter release, a major energy-requiring function of neurons, occurs in synapses at the distal ends of axons, many centimeters away from the cell body. It is hard to imagine that the steady-state level of glucose (and of glucose-derived ATP) would be high enough to drive solute transport in the far corners of a neuron. It is much easier to imagine that the required amounts of ATP are available because a large reservoir of ATP is brought to the site of usage by mitochondria that have traveled there (on cytoskeletal "railroad tracks" of F-actin or on microtubules and microfilaments [Morris and Hollenbeck, 1995; Simon *et al.*, 1995]) for that very purpose.

There is ample evidence that mitochondria are distributed preferentially at specialized sites of high metabolic activity. For example, when viewed in the electron microscope, mitochondria at the neuromuscular junction have been found in close proximity to the postsynaptic membrane (Lindgren and Smith, 1986), and mitochondria aggregate near the microvilli of locust photoreceptor cells after exposure to light (Sturmer *et al.*, 1995). Similarly, mitochondria in growing axons are preferentially distributed towards the growth cone (Morris and Hollenbeck, 1993). Finally, Hevner *et al.* (1992) have shown that mitochondrial cytochrome *c* oxidase activity colocalized with that for Na^+,K^+-ATPase in regions of monkey brain.

The preferential requirement for mitochondrial-derived ATP at the plasma membrane might also explain why a decrease in ATP synthesis of "only" 50% could impair neuronal function in a disease like MILS so severely: even though the overall, integrated level of ATP in the cell is down by half, at the plasma membrane the decline might be far more precipitous, especially if certain substrates (e.g., malate) were rate-limiting.

The idea that ATP is "compartmentalized" in the cell is not new, and is related to previous hypotheses regarding the subcellular compartmentation of creatine kinase in muscle (see, for example, Erickson-Viitanen *et al.*, 1982; Walliman *et al.*, 1992; Ishida *et al.*, 1994; Saks *et al.*, 1994). What has changed is that new tools are now available to test this hypothesis, especially as it relates to mitochondrial disease. In particular, the development of the luciferin–luciferase reaction, coupled with advances in molecular biology, should allow us to define the subcellular localization of ATP in cells (Aflalo and Segel, 1992; Mueller-Klieser and Walenta, 1993; Tamulevicius and Streffer, 1995). The ATP compartmentation hypothesis would predict that if analyses of this type were performed in cybrids harboring normal and mutated mtDNAs, one would see relatively high levels of "bioluminescent" ATP in the cytosol as compared to the plasma membrane. Put another way, one would also predict that the decline in the performance of an ATP-dependent ion pump (as measured by, for example, patch clamping) would be greater than the decline in the activity of an ATP-dependent cytosolic protein, such as a kinase.

Compartmentation of ATP might also explain why, as a general rule, cells in mitochondrial diseases are disabled, but often do not die. For example, mitochondrial myopathies are rarely accompanied by breakdown of skeletal muscle (muscle necrosis is rarely seen in biopsies and serum creatine kinase is usually normal) or by inflammatory reaction and connective tissue infiltration. Morphological examination of brain regions in patients with KSS or MELAS show little neuronal loss, even in those brain regions that are respiratorily deficient (i.e., COX-negative), and there are few macroscopic changes in the vasculature in MELAS. Similarly, cardiopathies cause muscle hypertrophy, but not muscle necrosis. Obviously there *are* morphological changes even in the above-men-

tioned tissues (Ban *et al.*, 1993), and there are clear exceptions to this assertion—most notably the neuronal loss in the striatal necrosis syndromes, the loss of retinal pigmented epithelium (causing pigmentary retinopathy) in KSS and MELAS (Rummelt *et al.*, 1993), and optic atrophy in LHON (although it is noteworthy that in rare instances, the blindness is *reversible*)—but to a remarkable degree, the devastating clinical effects of mtDNA mutations are not reflected by concomitantly severe effects at the morphological level in cells.

The supposition that mtDNA mutations affect the subcellular distribution of ATP might help explain why certain tissues are more affected than others in mitochondrial disease, but provides almost no insight at all regarding the problem of genotypic specificity (why different mtDNA mutations affect different cell types). That problem still awaits more incisive analysis.

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