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Review

Genetics and molecular pathogenesis of mitochondrial respiratory chain diseases

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Abstract. Dysfunction of the mitochondrial respiratory chain has been recognised as a cause of human disease for over 30 years. Advances in the past 10 years have led to a better understanding of the genetics and molecular pathogenesis of many of these disorders. Over 100 primary defects in mitochondrial DNA (mtDNA) are now implicated in the pathogenesis of a group of disorders which are collectively known as the mitochondrial encephalomyopathies, and which most frequently involve skeletal muscle and/or the central nervous system. Although impaired oxidative phosphorylation is likely to be the final common pathway leading to the cellular dysfunction associated with such mtDNA mutations, the complex relationship between genotype and phenotype remains largely unexplained. Most of the genes which encode the respiratory chain reside in the nucleus, yet only five nuclear genes have been implicated in human respiratory chain diseases. There is evidence that respiratory chain dysfunction is present in common neurological diseases such as Parkinson's disease and Huntington's disease. The precise cause of this respiratory chain dysfunction and its relationship to the disease process are unclear. This review focuses upon respiratory chain disorders associated with primary defects in mtDNA.

Key words. Mitochondrial DNA; mitochondrial encephalomyopathy; respiratory chain diseases; molecular genetics; oxidative phosphorylation.

Introduction

Many of the processes essential for eukaryotic cell survival are dependent upon adenosine triphosphate (ATP) [1]. It is therefore not unexpected that impaired activity of the mitochondrial respiratory chain should associate with cellular dysfunction leading to human disease. What has been surprising is the dramatic clinical diversity observed in patients with these disorders. Respiratory chain disease may develop at any time from the neonatal period to late adult life. Although tissues with a high demand for oxidative phosphorylation such as brain and skeletal muscle are frequently affected, virtually any tissue can be involved. The clinical phenotypes may therefore vary widely. For example, patients may present with a devastating fatal lactic acidosis in the neonatal period or a mild proximal myopathy (limb weakness) in late adult life [2–5]. There have been dramatic advances in defining the molecular genetic basis of many respiratory chain disorders in the last 10 years. These advances have mainly been in the area of mitochondrial DNA (mtDNA) mutations [6, 7]. Over 100 mtDNA mutations are now described, and the use of cell culture models has facilitated the elucidation of their molecular pathogenesis.

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The overwhelming majority of mitochondrial respiratory chain proteins are not encoded in mtDNA but by nuclear genes [5]. It is therefore surprising that to date only two nuclear gene mutations have been identified, although nuclear gene defects are strongly implicated in three other respiratory chain disorders [8–11, 90]. This is at least in part because the mitochondrial genome is small (16,569 bp) and relatively easier to analyse [12].

Although much is now known about these mitochondrial and nuclear gene defects, a precise molecular understanding of the genotype-phenotype correlations observed remains an important challenge. For example, it remains unclear why the same mtDNA point mutation may associate with dramatically different clinical phenotypes ranging from diabetes mellitus alone to a devastating central nervous system (CNS) disorder characterised by recurrent strokes and progressive dementia [7].

In this article we review some of the basic characteristics of the respiratory chain and mtDNA before discussing some important aspects of the genetics and molecular pathogenesis of mitochondrial respiratory chain diseases. We have focused upon respiratory chain disorders associated with primary defects in mtDNA.

The mitochondrial respiratory chain: structure and function

The respiratory chain is located in the inner mitochondrial membrane (fig. 1). It comprises four multisubunit enzyme complexes: complex I (nicotinamide adenine dinucleotide ((NADH)-ubiquinone oxidoreductase); complex II (succinate-ubiquinone oxidoreductase); complex III (ubiquinol-cytochrome c oxidoreductase) and complex IV (cytochrome c oxidase). In addition, there are two mobile electron carriers: ubiquinone and cytochrome c. Along with ATP synthase (complex V) these complexes and mobile electron carriers comprise the oxidative phosphorylation system [5]. Reducing equivalents are transferred to the respiratory chain via NADH and flavin adenine dinucleotide ($FADH_2$) and finally to molecular oxygen to form water. The respiratory chain transfers electrons down a stepwise energy gradient. The energy released is used by complexes I, III and IV to pump protons across the inner mitochondrial membrane into the intermembrane space. An electrochemical proton gradient is thereby generated across the inner membrane ($\Delta\psi$ [mitochondrial membrane potential] + ΔpH [proton gradient]). This proton motive force (PMF) is harnessed by ATP synthase (complex V), which is essen-

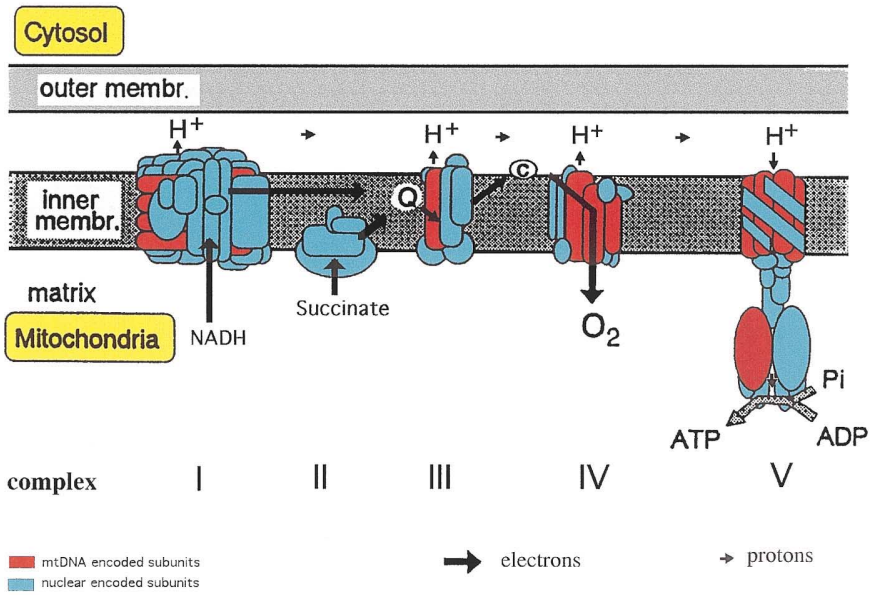

Figure 1. The mitochondrial respiratory chain located on the inner mitochondrial membrane. Q, quinone; c, cytochrome c. The direction of electron flux and the proton-pumping capability of complexes I, III and IV are indicated by arrows.

Table 1. Composition of the respiratory chain and oxidative phosphorylation system.

Complex	Total no. subunits	No. subunits encoded by mtDNA
I	>42	7 (ND 1-6) ND4L
II	4	0
III	11	1 cytochrome b
IV	13	3 COX I, II, III
V	16	2 ATPase 6 and 8

Complex II subunits are all encoded in the nuclear genome, as are the majority of the subunits of the other complexes.

tially an ATP-hydrolysing pump, in the direction of ATP synthesis from adenosine diphosphate (ADP) and inorganic phosphate [13]. The mitochondrial ATP generated in the mitochondrial matrix by this process is exchanged for cytosolic ADP across the inner mitochondrial membrane by the adenine nucleotide translocator [14]. Reactive oxygen species are generated as a consequence of normal respiratory chain function, and it has been shown that inhibition of the respiratory chain results in an increase in the generation of such reactive oxygen species [15]. The major consequences of respiratory chain dysfunction are therefore a decrease in cellular ATP and an increase in toxic reactive oxygen species, both of which may impair cell function.

With the exception of complex II subunits (which are entirely nuclear-encoded) each of the respiratory chain complexes contain both nuclear and mitochondrially encoded subunits (table 1) [5]. The nuclear encoded subunits are synthesised on cytoribosomes and subsequently imported into the mitochondria, facilitated by tagging with an N-terminal presequence, where they are processed and assembled with their mitochondrially encoded counterparts into functional enzyme complexes. A number of different nuclear-encoded proteins such as the chaperonins and heat shock proteins are important in facilitating the import of nuclear-encoded respiratory chain subunits into the mitochondrion [16]. It is evident that an elaborate interplay between nuclear and mitochondrially encoded proteins is essential for the biogenesis of a functional respiratory chain. It follows that dysfunction of the respiratory chain may result from disruption of either nuclear or mitochondrial genes or both.

mtDNA

Organisation

Mitochondria are unique among cellular organelles in that they possess their own DNA [12]. MtDNA is a closed circle double-stranded molecule of 16,569 nucleotide pairs (fig. 2). It is a highly compact genome with a small noncoding region (the displacement-loop or D-loop) which is estimated to be 1123 bp in length and contains the major control elements for mtDNA replication and transcription (fig. 3). mtDNA only contains 37 genes: 22 transfer RNA genes, 2 ribosomal RNA genes and 13 polypeptide-encoding genes. The ribosomal RNA (rRNA) and transfer RNA (tRNA) genes in combination with nuclear-encoded factors allow mitochondria to translate the mitochondrially encoded polypeptides on mitochondrial ribosomes. The mutation rate of mtDNA is several times higher than that of nuclear DNA [17]. This is likely to be due to a combination of factors which include the absence of protective DNA-binding proteins like histones, the absence of an effective DNA repair mechanism and the hostile free-radical environment of the mitochondrial central matrix where the mtDNA is located.

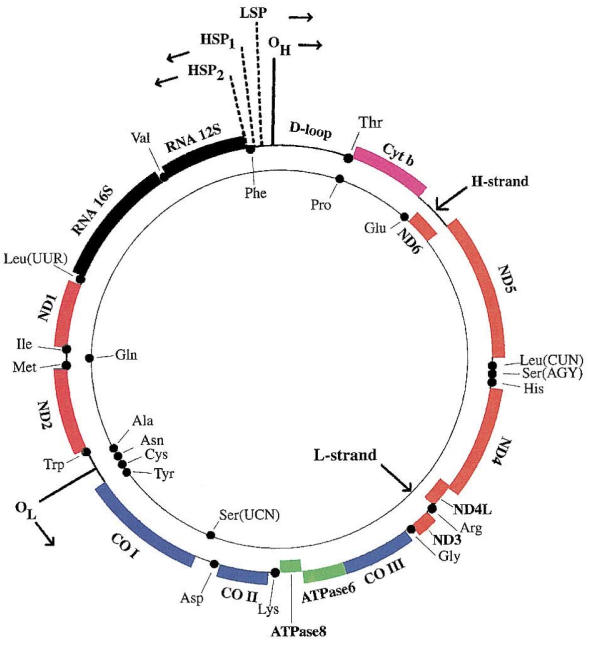

Figure 2. Human mitochondrial DNA showing the double-stranded circular structure. The 22 tRNA genes are indicated by black circles, the 2 rRNA genes by black bars and the protein-coding genes by coloured bars. The origins of light- and heavy-strand replication are indicated by O_H and O_L, respectively, the light-strand promotor by LSP and the two heavy-strand promotors by HSP_1 and HSP_2. Complex I (NADH) subunits are indicated by ND (1, 2, 3, 4, 4L, 5, 6). Cytochrome b is indicated by Cyt b. Complex IV (CO) subunits are indicated by CO (I, II, III). Complex V subunits are indicated by ATPase (6, 8).

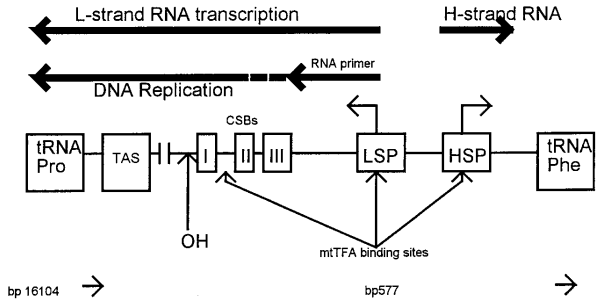

Figure 3. DNA replication is primed by the synthesis of a short strand of RNA beginning at the LSP. The transition to DNA occurs between CSBs II and III. The 3′ end of the D-loop maps to the TASs. mtTFA binds within each promotor region (LSP and HSP) upstream of the transcription start sites (see text.)

Replication

Each strand of mtDNA is replicated from a separate replication origin [18, for detailed review of mtDNA replication]. The H-strand replication origin (O_H) is situated in the D-loop, and the light-strand origin (O_L) is situated in a cluster of five tRNA genes (fig. 2). Replication is initiated at O_H by the synthesis of a short primer strand of RNA from the light-strand promoter (LSP) (see fig. 3). This strand of RNA is synthesised when a mitochondrial transcription factor (mtTFA) binds to the LSP. The change from RNA primer synthesis to DNA (known as 7S DNA) synthesis occurs between conserved sequence blocks II and III (CSB II and III) and a nuclear encoded endoribonuclease, termed RNase MRP (for mitochondrial RNA processing), seems to be essential for this transition to occur [19, 20]. Replication proceeds around the L-strand displacing the parental H-strand. L-strand replication begins when O_L is exposed and proceeds back along the free H-strand. mtDNA replication may therefore be described as an asynchronous strand-displacement process. Whether L-strand replication (H-strand synthesis) represents extension of arrested 7S DNA or requires a new round of initiation and synthesis through the D-loop region is unknown. Most 7S DNA synthesis arrests in the region of the termination-associated sequences (TASs). The 7S DNA has been demonstrated to be highly unstable with a high turnover. The commitment to replication and presumably its control are therefore dependent upon mtTFA and RNAse MRP. These factors are both nuclear-encoded. The enzyme γ-polymerase is responsible for mtDNA replication. The gene for γ-polymerase maps to chromosome 15q24–25 and has been cloned recently. Sequence analysis of this gene revealed a $CAG_{(n=10)}$ repeat at the 5′ end of the complementary DNA (cDNA) [21]. In contrast to disease-associated CAG repeats, this repeat appears to be stable on transmission (I. P. Nelson and M. G. Hanna, unpublished observations).

Alterations in replicational activity is one level at which mitochondrial gene expression can be controlled. It has been demonstrated that the concentration of transcripts of mitochondrial genes varies in proportion to the mtDNA concentration and to the oxidative capacity in rabbit striated muscle after electrical stimulation in vivo [22]. Similarly, Cantatore and colleagues demonstrated that the developmentally related increase in mitochondrial gene expression in rat hepatocytes was achieved by increasing cellular mtDNA content [23]. These observations are surprising, since available evidence suggests that normally transcription is not rate-limiting [24].

Transcription

Transcription begins from the promotors in the D-loop region (fig. 3). These promotors require binding of mtTFA for initiation [18, 25, 26]. mRNA synthesis then proceeds around the mtDNA molecule, producing a polycistronic transcript. This transcript is subsequently cleaved at specific sites which often coincide with tRNA genes to release tRNA, rRNA and messenger RNA (mRNA) molecules. Nuclear-encoded enzymes are thought to be responsible for this processing [27]. Mitochondrial mRNAs are not capped with 7-methyl guanosine, but they do have a 3′ polyadenylated tail, which is added after cleavage and is essential for translation [12].

There are two overlapping transcription units on the H-strand which allow rRNA species to be transcribed at 15–60 times higher rates than mRNAs. This is achieved by transcription termination activity within the $tRNA^{Leu(UUR)}$ gene. Termination may be mediated by a protein fraction mitochondrial transcription termination factor (mTERF) [28].

In addition to transcriptional control mediated by mTERF, there is evidence that differential gene expression may be mediated at the level of transcription by differences in RNA stability. For example, despite marked differences in the rates of transcription of the three transcriptional units (rRNA: L-strand: H-strand/ 25:10:1), the steady-state levels of tRNAs are very similar. Attardi and colleagues have suggested that this is at least in part due to the availability of factors capable of stabilizing newly formed tRNA molecules [29]. The observation that the mRNAs derived from processing of the whole H-strand polycistronic transcript are present in quite different steady-state amounts suggests that differential mRNA turnover rates may also be involved in influencing differential gene expression.

Translation

Translation of mtDNA-encoded peptides occurs on mitochondrial ribosomes, but relatively little is known about the details of this process. As with cytosolic translation, a variety of nuclear-encoded factors are involved [24]. The mitochondrial genetic code differs from the nuclear code (UGA:Trp, AUA:Met, AGA or AGG:Stop) [12]. mtDNA only codes for 22 tRNA molecules. This economy is achieved by single tRNAs recognising four codons coding for a single amino acid.

The sequences of mammalian mitochondrial tRNAs are unusual in that they lack several of the so-called invariant and semiinvariant nucleotides when compared with their cytoplasmic 'classical' counterparts [a schematic representation of the theoretical two dimensional structure of a tRNA leucine (UUR) molecule is shown in fig. 4]. They also differ in stem and loop sizes. Gadaleta and colleagues have compared mitochondrial tRNA genes from five different species (human, bovine, mouse, rat and *Xenopus*) and made the following conclusions: (i) the most conserved tRNA genes are those that decode the most commonly used amino acids, (ii) the anticodon loop is the most conserved region, (iii) the TΨC loop is the most variable region and (iv) the variable loop is always short [30]. The precise three-dimensional structure of human mitochondrial tRNA molecules and how this is altered by pathogenic mutations is presently unknown. There is evidence that some pathogenic tRNA gene point mutations impair aminoacylation of the tRNA molecule [31, 32].

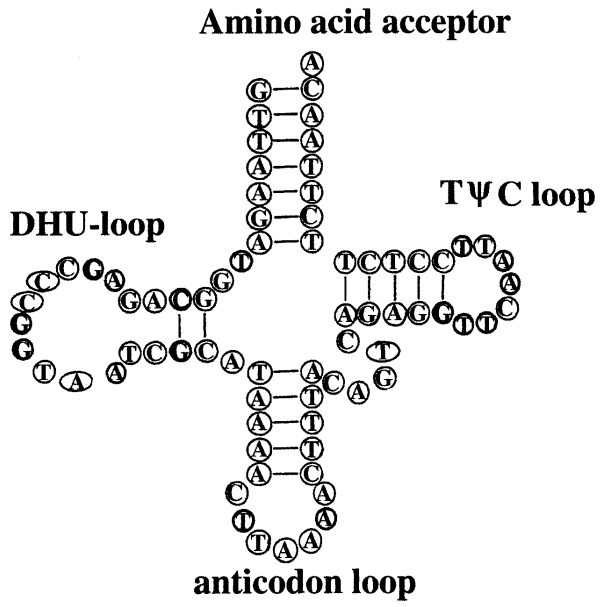

Figure 4. Diagram illustrating the predicted secondary structure of the tRNA leucine (UUR) molecule. The four major domains are the amino acid acceptor stem, the dihydrouridine loop (DHU-loop), the anticodon loop and the TΨC loop.

It is likely that translational control represents a third level at which mitochondrial gene expression can be influenced, in particular, differences in translational efficiency of mRNA. In HeLa cells it has been shown that there is a greater than 10-fold variation in the rates of synthesis of different mitochondrially encoded proteins which is not reflected in the steady-state levels of mRNA, suggesting that the efficiency of translation of different mRNAs may vary [33]. The basis for this apparent variation in translational efficiency is uncertain. It appears not to be due to the presence of rarely used codons. It is possible that differences among the various mRNAs' degree of secondary structure and/or accessibility of the initiator codon may be important. Alternatively, as yet unidentified nuclear factors may be important [33]. Bourgeron and colleagues (1993) have presented evidence that recovery of respiratory chain function in transformed lymphocytes harboring deleted mtDNA may be due to an increase in translational efficiency [34].

Unique characteristics of mtDNA genetics

There are four important principles in mitochondrial genetics which are pertinent to understanding the inheritance and molecular pathogenesis of diseases associated with some primary defects in mtDNA. These are (i) maternal transmission, (ii) mitotic segregation, (iii) heteroplasmy and (iv) the threshold effect.

Maternal transmission. mtDNA is maternally inherited. Most, but not all, point mutations in mtDNA exhibit maternal inheritance [36, 48]. Hence, diseases associated with point mutations of mtDNA may exhibit a matrilineal pattern of inheritance. This is probably because the midpiece of the spermatozoan, which is the only part of the sperm containing mitochondria (and therefore mtDNA), does not penetrate the ovum [35, 36]. However, some have controversially challenged this hypothesis [115]. It follows that males cannot transmit diseases which are associated with point mutations in mtDNA. Although it can be predicted with confidence that the majority of disease-associated mtDNA point mutations will be transmitted from a female to all her offspring, this does not necessarily mean that all the offspring will develop disease. The propensity of a given offspring harbouring a disease-causing mutation to develop disease, that is the disease penetrance, is a complex phenomenon which is not yet fully understood for any of the mtDNA point mutations. Some of the important variables in determining penetrance include segregation, degree of heteroplasmy and the threshold effect, and are described below.

In contrast to most point mutations of mtDNA, single large-scale deletions are virtually never maternally inherited. It is not clear why this should be the case, since deleted molecules have been detected in oocytes of women with mtDNA deletion-associated disease [37]. Large shifts in the proportion of a heteroplasmic sequence variant (be it a polymorphism or a pathogenic mutation) have been observed between generations. For example, studies on Holstein cows have shown that there can be a complete change from one sequence variant to another within a single generation (so-called allele switch) [38, 39]. This is somewhat unexpected when one considers that oocytes have a very high mtDNA copy number, which has been estimated to be over 10^5 [40, 41]. The concept of the mitochondrial genetic bottleneck has been proposed as a mechanism to explain such large shifts. It is suggested that at some point during oogenesis a few mtDNA molecules are selected which actually contribute to the next generation. The precise size and timing of this bottleneck have been the focus of much recent research. Animal studies have indicated that most of the segregation has occurred by the time oocytes are fully mature [42, 43]. The mechanism of the bottleneck is not yet understood [42].

Segregation. Studies of heteroplasmic cell lines have provided evidence for the concept of mitotic segregation [44]. This refers to the fact that both mtDNA replication and mitochondrial division are considered to be stochastic processes unrelated to the cell cycle or to the timing of nuclear replication. Thus, a dividing cell which is heteroplasmic may potentially donate different proportions of mutant mtDNA to its daughter cells. However, there is evidence that replication and partitioning of mtDNA may in fact not be entirely random processes [43, 45]. For example, recent studies in mice heteroplasmic for mtDNA polymorphisms have shown tissue-specific selection for some heteroplasmic polymorphisms in some tissues [43]. This indicates that in some situations tissue-specific factors are important in determining the distribution of a given heteroplasmic mtDNA variant within an organism [43]. The nature of such interactions between nuclear and mtDNA remains to be determined.

Heteroplasmy. Individual mitochondria contain 2–10 copies of mtDNA [40, 41]. Since individual cells contain many mitochondria, it follows they contain a large number of mtDNA molecules. In a healthy individual or cell the DNA sequence of all the copies of mtDNA molecules is identical—a state known as homoplasmy. Intraindividual variation in the sequence of mtDNA molecules is termed heteroplasmy. In humans heteroplasmy is generally deleterious and associated with pathogenic mutations of mtDNA. Heteroplasmy for pathogenic mtDNA mutations has been demonstrated at the level of the cell, but it remains uncertain whether heteroplasmy exists within a mitochondrion.

Threshold effect. The threshold effect refers to the observation that for many pathogenic mtDNA mutations which have been studied, there is a critical proportion of the mutant form which must be exceeded before respiratory chain dysfunction ensues. It appears that different types of primary mtDNA mutations may have different thresholds for expression [46–48, 50, 51]. Furthermore, there is evidence that the threshold for expression of the same mtDNA mutation may differ in different cell types [46, 47].

From a clinical viewpoint a better understanding of the mechanisms involved in influencing the transmission of heteroplasmic mtDNA defects between generations (i.e. the bottleneck), and in controlling their tissue segregation and threshold for expression, will be essential if genetic counselling for these disorders is to become possible.

Respiratory chain dysfunction and human disease

Mutations in mtDNA and the mitochondrial encephalomyopathies

Since the first primary defect in mtDNA was identified in 1988, almost 100 different pathogenic defects have been reported [6, 7]. These fall broadly into four main groups: (i) large-scale rearrangements (mainly single deletions of mtDNA, in which large segments of the mitochondrial genome, usually several thousand base pairs, are deleted although large-scale partial duplications, and microdeletions are now also reported) [6, 7, 56, 57, 79, 106]; (ii) tRNA gene defects (mainly point mutations, but insertions and deletions of single base pairs are reported) [6, 7, 66, 105]; (iii) protein-coding gene point mutations [56, 113, 111]; and (iv) rRNA gene point mutations [107].

Since mtDNA is highly polymorphic, it is not uncommon to identify changes in the mtDNA sequence between two individuals, especially if they are of different ethnic origins. Caution must therefore be exercised before ascribing pathogenic status to any change identified. A stringent list of criteria which would indicate pathogenic status might include the following: (i) the mutation is heteroplasmic, that is it coexists with wild-type within the same individual; (ii) the mutation occurs at a position within the mitochondrial genome which is conserved through evolution, suggesting functional importance; (iii) the mutation is not present in a large panel of ethnically matched healthy control samples; (iv) the mutation is shown to have arisen independently in association with disease in unrelated families; (v) expression studies, such as the rho zero cell model, demonstrate a direct association between the proportion of mutant mtDNA and a functional consequence, such as impaired intramitochondrial protein synthesis (for tRNA gene

defects and large-scale rearrangements) or impaired respiratory chain biochemistry (for all types of mtDNA mutations). While a mutation which met all the above criteria would certainly be regarded as pathogenic, it should be noted that there are some pathogenic mutations which do not fulfil all criteria. For example, it is often difficult to demonstrate heteroplasmy for the three primary pathogenic mutations which cause Leber's hereditary optic neuropathy (LHON). Functional expression studies probably represent the most useful way of confirming pathogenicity, although such studies are often difficult to perform.

The range of clinical phenotypes which are described in association with these different primary mtDNA defects is quite remarkable. (A comprehensive list of pathogenic mtDNA mutations and associated phenotypes is provided in reference [7].) Respiratory chain dysfunction has been documented in most of these disorders, although there is often no clear correlation between the site of the biochemical defect and the clinical phenotype [52, 53]. It had been hoped that the advent of specific mtDNA defects might allow clear genotype-phenotype correlations to be made. To a limited extent this hope has turned out to be true, but there are many exceptions to this rule [54]. For example, whilst the A3243G mutation in the tRNA gene for leucine (UUR) is the commonest cause of the mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS) phenotype, this mutation can associate with completely different phenotypes such as chronic progressive external opthalmoplegia (CPEO). Conversely, the MELAS phenotype may associate with a number of other mtDNA defects. Other mtDNA defects are more consistently associated with specific phenotypes. This applies to the mutations which cause LHON and NARP. Some of the commonly recognised phenotypes are outlined below.

A common histopathological feature in the skeletal muscle of patients with defects of mtDNA are so-called ragged red fibres (RRF). These represent areas of focal mitochondrial proliferation. The precise trigger for mitochondrial proliferation is not known, but it may represent an attempt to compensate for impaired respiratory chain function within a muscle fibre segment. A recent study implicates neurotropin 4 in mitochondrial proliferation [55]. Such mitochondrial proliferation is more commonly seen in those primary mtDNA defects which affect tRNA genes (such as tRNA gene point mutations and deletions rather than protein-coding mutations). Elevation of the plasma lactate is also commonly found in patients with respiratory chain disease.

Commonly recognised mtDNA mutation-associated phenotypes

Kearns-Sayre syndrome (KSS). This disorder is most frequently associated with single deletions of mtDNA [7]. The core clinical features are a progressive weakness of the muscle which move the eyes (chronic progressive external ophthalmoplegia, CPEO) and a pigmentary retinopathy, both of which develop before the age of 20 years. Patients usually also have at least one of the following: a cerebellar syndrome, cardiac conduction block or elevated cerebrospinal fluid protein. A variety of other features are variably present and include deafness, limb weakness and renal and endocrine dysfunction. Skeletal muscle histochemistry typically shows RRFs which often show reduced COX (cytochrome c oxidase) activity in cases associated with single deletions. There is progressive deterioration, and premature death is usual [7, 56, 57].

CPEO. This is a relatively benign disorder which typically develops either in teenage years or in later life. There is progressive weakness of the extraocular muscles, eventually leading to a complete ophthalmoplegia. Most cases are sporadic and are commonly associated with single deletions of mtDNA, but it may also occur in association with some tRNA gene point mutations [7].

MELAS. MELAS is a common maternally inherited disorder often resulting in severe disability and premature death. Patients experience strokelike episodes often before the age of 40 years, an encephalopathy characterised by dementia and seizures, and most cases have RRFs on muscle biopsy. A range of additional features may be present, including recurrent vomiting, migrainelike headaches, limb weakness and short stature. It is most commonly associated with a point mutation in the tRNA gene for leucine (UUR) but is genetically heterogeneous [6, 7, 66, 79].

Myoclonic epilepsy with ragged red fibres (MERRF). The core clinical features of MERRF are myoclonus, ataxia and seizures. Limb weakness of varying severity may be present, and the muscle biopsy shows ragged red fibres. Other features which may occur include dementia, deafness, optic atrophy, short stature and neuropathy. It is most commonly associated with point mutations in the tRNA gene for lysine [6, 7, 64].

Neurogenic muscular weakness, ataxia and retinitis pigmentosa (NARP). Patients develop progressive muscle weakness secondary to a motor neuropathy in combination with ataxia and visual impairment secondary to retinal degeneration. It is most commonly associated with a point mutation in the adenosine triphosphatase (ATPase) 6 gene [7, 111].

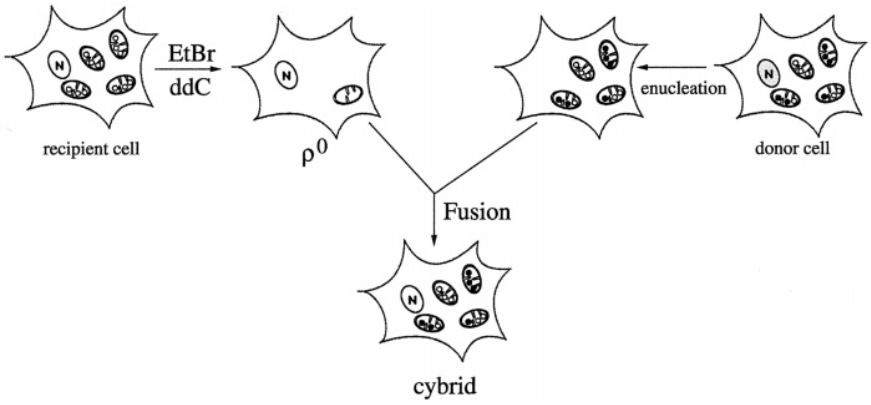

Figure 5. Diagram illustrating the generation of transmitochondrial cybrids. The recipient cells (usually a transformed cell line) are depleted of mtDNA by exposure to ethidium bromide (EtBr) or 2′,3′-dideoxycytidine (ddC). The resulting cells, which contain no mtDNA, are termed rho zero. Rho zero cells are fused with donor cells (usually myoblasts of fibroblasts from a patient harbouring a pathogenic mtDNA mutation, indicated by black circle in diagram) which have been enucleated by exposure to cytochalasin B (enucleated cells are known as cytoplasts). The resulting transmitochondrial cybrids allow the mtDNA mutation of interest to be studied in a constant nuclear background.

LHON. This mitochondrial disease results in severe subacute loss of central vision, mainly in young adult males between the ages of 18 and 30 years. It is one of the commonest causes of blindness in otherwise healthy adult males. The resulting blindness is usually permanent. It is most commonly associated with a point mutation in the ND4 protein-coding subunit at position 11,778. Two other protein-coding gene point mutations at positions 3460 and 14,484 account for the majority of patients not harbouring the 11,778 mutation. For reasons which remain unclear, males are much more likely to develop blindness than females despite harbouring similar proportions of the mutant mtDNA [58, 59].

Leigh's syndrome. Leigh's syndrome (subacute necrotising encephalomyopathy) is a progressive neurodegenerative disorder that usually has its onset in infancy or early childhood, although adult onset cases are recognised. Brain imaging has a very characteristic appearance with bilateral high signal on T2 images in the thalamus extending down into the brainstem. With few exceptions, the prognosis is poor. It is associated with the T8993G NARP mutation in some cases. However, most cases associate with complex IV or pyruvate dehydrogenase or less commonly with complex I deficiency, and are believed to have a nuclear genetic basis [60, 63].

A large number of other clinical phenotypes have now been described in association with primary defects of mtDNA [7]. It is noteworthy that whereas some mtDNA defects associate with multisystem disease (e.g. KSS) or widespread involvement of neurological tissues (e.g. MELAS, NARP), others are remarkably tissue-specific. For example, in the overwhelming majority of patients with LHON, the disease is confined to the optic nerve alone. This is despite the fact that the disease-causing mutation is probably virtually homoplasmic in all tissues.

Molecular pathogenesis of disease associated with mtDNA mutations

An important advance in the elucidation of the molecular pathogenesis of disease associated with mtDNA defects has been the use of the rho zero cell model [61]. These workers generated human immortal tumour cell lines which were completely depleted of their mtDNA (but not mitochondria) by long-term exposure to ethidium bromide, which inhibits mtDNA replication. The mitochondria in rho zero cells are able to maintain a membrane potential and are able to produce some ATP by glycolysis. However, since they do not have an intact respiratory chain, they cannot respire. They require glucose-containing medium in order to grow normally. The medium also has to be supplemented with uridine and pyruvate. In the absence of a functional respiratory chain, pyrimidine biosynthesis is impaired and uridine compensates for this. Pyruvate is required to reoxidise NADH, which would otherwise be undertaken by a functional respiratory chain.

Rho zero cells can be fused with enucleated cells (cytoplasts) containing mitochondria with mtDNA mutations of interest to generate transmitochondrial cybrids. Such cybrids can be selected for in pyruvate-supplemented uridine-free medium. This system allows the study of the effects of different proportions of mutated

mtDNA on mitochondrial transcription, translation and respiratory chain biochemistry in a constant nuclear background (illustrated in fig. 5). It has been used to study the consequences of several mtDNA transfer ribosomal nucleic acid (tRNA) gene point mutations, as well as deletions and the common mutations in protein-coding subunits which cause LHON.

Additional cell systems have been developed which allow untransformed patient-derived cells to be depleted of their mtDNA. Such systems facilitate the identification of nuclear genes which either cause respiratory chain disease or modulate the effect of primary mtDNA mutations [62, 63].

Molecular pathogenesis of MERRF. The commonest mtDNA gene defect associated with this phenotype is the A8344G point mutation in the tRNA gene for lysine [64]. Studies on transmitochondrial cybrids and primary muscle cell cultures generated from patients harbouring the A8344G mutation have provided important insights in the cellular molecular pathogenesis of disease associated with this mutation. In primary myoblast cultures the A8344G mutation results in impaired synthesis of all mitochondrially encoded peptides, as might be predicted for a tRNA gene point mutation. The proportion of the mutation required to induce translational impairment appeared to be less in myoblasts than in differentiated myotubes, possibly because of the lower mtDNA copy number per mitochondrion in the former [46, 47]. Initial rho zero cell studies also clearly demonstrated that cybrids harbouring virtually homoplasmic mutant A8344G exhibited a marked decrease in translation and extremely low levels of respiration. In contrast, wild-type cybrids generated from the same heteroplasmic patient exhibited entirely normal translation and respiratory chain function [50].

More recently similar studies on mutant (A8344G) transmitochondrial cybrids have identified several abnormal peptides in addition to revealing a marked reduction in translation. Evidence has been provided that some of these abnormal peptides represent truncated forms of the normal mtDNA-encoded respiratory chain peptides. The same group proposed that the decrease in the rate of synthesis of mitochondrial peptides correlated with the number of lysine residues in the peptide. It was suggested that there was an increased probability of premature protein chain elongation termination at each lysine codon. There is no evidence that impaired transcription contributes to the pathogenesis of disease associated with the A8344G point mutation [31, 47, 65]. It seems that the level of aminoacylated tRNA lysine is 50–60% lower in mutant cybrids compared with wild-type. In other words the premature termination in translation observed seems to be due to a decrease in the levels of the charged tRNA molecules. A small decrease in the aminoacylation has also been reported with the A4317G mutation, but such a mechanism is not yet reported in any other pathogenic tRNA mutations [32].

Molecular pathogenesis of MELAS. The other common tRNA gene point mutation is in the tRNA gene for leucine (UUR) at position 3243 [66]. In addition to being located within the tRNA gene for leucine (UUR), this mutation is located within an important regulatory region. This is a tridecamer sequence which binds the transcription termination factor mTERF. As already described, binding of mTERF at this site serves to selectively increase the rate of transcription of 12S and 16S rRNA [28]. Therefore, in addition to impairment of the tRNA molecule function, impairment of mTERF binding was also a candidate process which might be impaired in the presence of the A3243G mutation. Whilst mTERF binding has been shown to be impaired in the presence of the A3243G mutation in vitro, there is no evidence that this is in fact the case in vivo [67–69]. Cybrid lines harbouring greater than 95% A3243G mutation exhibited a generalized decrease in translation and a severe respiratory chain deficiency [68]. A small increase in the steady-state level of an abnormal RNA transcript, termed RNA 19, was observed corresponding to $16SRNA + tRNA^{Leu(UUR)} +$ ND1. It was concluded that the A3243G mutation caused loss of respiratory chain activity through a combination of impaired translation and possibly transcript processing. It was suggested that the small amount of RNA 19 might have a significant effect on translation if it interacted with ribosomes in such a way as to render them functionally deficient [68, 71]. Suomalainen and colleagues (1993) suggested that there may be a reduced capacity to compensate for defective translation in the presence of the 3243 mutation [70].

Molecular pathogenesis of disease associated with duplication and deletions of mtDNA. Hayashi and colleagues 1991 demonstrated that cybrid clones harbouring greater than 65% of a 5196-bp deletion exhibited a rapid decline of overall mitochondrial translation consistent with the loss of tRNA genes encompassed within the deletion. With proportions of mutant mtDNA less than 65% there was evidence of complementation, since undeleted reading frames appeared to be translated normally and a fusion protein was observed. Taken together these data indicate that the threshold for biochemical expression for this deletion was greater than 65% and that wild-type and mutant mtDNAs must coexist within the same organelle, allowing complementation to occur at subthreshold proportions of mutant mtDNA [51].

In addition to single deletions of mtDNA some patients harbour partial duplications. The precise significance of such duplication is unclear. There is evidence that they merely represent nonpathological intermediates [72, 73].

Table 2. Genetic classification of respiratory chain diseases.

Genome	Common phenotype	Reference
mtDNA		
Large-scale rearrangement	CPEO KSS Pearson's syndrome	56, 106
tRNA gene point mutations		
tRNA leucine (UUR) A3243G	MELAS CPEO DM DM and deafness CPEO and CM MERRF KSS	66
tRNA lysine A8344G	MERRF	64
rRNA gene point mutations		
12SrRNA A1555G	nonsyndromic deafness antibiotic-induced deafness	107, 111
Protein coding gene point mutations		
ND4 G11778A	LHON	56
ATP6 T8993G	NARP	113
	Leigh's syndrome	110
Nuclear DNA		
Complex I gene		
homozygous 5-bp deletion		
18-kDa (AQDQ) subunit (ch 5)	fatal encephalopathy	9
Complex II gene		
Fp subunit point mutation (ch5p15)	Leigh's syndrome	8
Multiple-deletion mtDNA (secondary to unidentified nuclear gene defect) linkage established:		
10q23.3–q24.3	CPEO	109
3p14.1–21.2	CPEO	114
22q13.32	MNGIE	116

The commonest phenotypes associated with each genotype have been listed. Only the first report is sited. A full list of genotype-phenotype correlations is given in [7]. In the case of the A3243G mutation, which is the mtDNA mutation exhibiting the most marked phenotypic variation, a more extensive list has been included to complement the discussion given in the text. CM, cardiomyopathy; DM, diabetes mellitus; MNGIE, mitochondrial neurogastrointestinal encephalomyopathy. All other abbreviations used in this table are defined in the text. COX-deficient LS and the mtDNA depletion syndrome are likely to be nuclear gene disorders but are not included since neither linkage data nor the responsible genes are defined.

This is supported by one study in which cybrids which were virtually homoplasmic for a partial duplication did not exhibit significantly impaired respiratory chain function [74].

Molecular pathogenesis of protein-coding gene mutations. The commonest point mutation which causes the NARP phenotype (T8993G) changes a highly conserved leucine for arginine in the ATPase 6 subunit which is a component of the proton channel of complex V. Cybrid and lymphoblast mitochondria studies have indicated that when present in virtually homoplasmic mutant proportions, this mutation reduces mitochondrial respiration and ATP production [75]. While the pathogenic role of the three common LHON-associated mutations is beyond doubt, the precise molecular consequences of these mutations when expressed in rho zero cell systems is less clear. The 3460 mutation has consistently been associated with a defect in complex I activity, whereas studies on the 11,778 and 14,484 mutations have been contradictory, some finding decreased complex I activity, others finding normal activity. A recent in vivo phosphorous magnetic resonance spectroscopy study of skeletal muscle from patients with LHON indicated that all three common mutations impaired respiratory chain function, but this was subtle with the 3460 mutation [76–78].

Unresolved issues in mtDNA-associated disease: the phenotype-genotype correlation

A great deal of information has been obtained by using the rho zero cell model to express defects of mtDNA in a constant nuclear background. Many aspects of the molecular mechanisms of disease associated with the commonest mtDNA mutations (A3243G, A8344G and deletions) have been elucidated.

While different mtDNA mutations may induce translation failure by different mechanisms, the consequence remains the common theme of impaired respiratory chain function. It is therefore unclear why certain genotypes should frequently associate with certain phenotypes. Conversely, the observation that certain genotypes, such as the A3243G mutation, can associate with a wide variety of phenotypes also requires explanation (see table 2). This complex and poorly understood relationship between the genotype and the phenotype is likely to depend on a large number of other factors. These probably include the tissue distribution of the mutant, the overall mtDNA copy number, the mtDNA haplotype, the ability of the translational machinary to compensate for a given mechanism of translation failure and perhaps the capacity of mitochondria to fuse and thereby facilitate intramitochondrial complementation [80–84]. Immunological factors have also been suggested [85]. It seems likely that many of these variables are influenced by nuclear genes. While the tissue distribution and proportion of mutant mtDNA may be important, these factors cannot explain the observations made in relation to mitochondrial nonsyndromic deafness (associated with the 12S RNA 1555 mutation) and LHON. Both of these disorders are highly tissue-specific, and yet available evidence indicates that all tissues are virtually homoplasmic for the mutant form. For the nonsyndromic deafness phenotype a 'two-hit' model has been proposed. That is, the mutation in homoplasmic form is necessary but not sufficient to cause the disease. A second 'hit', namely an unidentified nuclear gene defect or exposure to aminoglycosides, is needed for the disease phenotype to occur [107, 108, 112]. Further study is needed to achieve a better understanding of the relationship between genotype and phenotype.

Nuclear-encoded mitochondrial proteins and respiratory chain disease

Compared with studies on mtDNA defects, much less is known about nuclear gene defects which result in respiratory chain dysfunction. There is now strong evidence that at least five distinct respiratory chain diseases are due to defects in nuclear genes. For two of them the precise gene defect has been identified (one case of complex I deficiency and two sibs with complex II deficiency), for one genetic linkage has been established but identification of the gene is awaited (multiple mtDNA deletion disorders) and for two disorders complementation studies have provided strong evidence that these are nuclear gene disorders (mtDNA depletion syndrome and COX-deficient Leigh's syndrome) [8–11].

Isolated complex II deficiency

The first nuclear gene defect reported to cause respiratory chain dysfunction was in the flavoprotein subunit of complex II. This was a homozygous Arg554Trp substitution in the Fp subunit on chromosome 5p15 identified in two sibs with Leigh's syndrome and a severe deficiency of complex II activity. The mutation was shown to impair complex II catalytic activity in a yeast strain transformed with the Fp cDNA [8].

Isolated complex I deficiency

A second nuclear gene defect has been identified recently in a case of complex I deficiency. The male infant had a fatal encephalopathy caused by a severe deficiency of complex I. A homozygous 5-bp duplication in an 18-kDa complex I subunit gene on chromosome 5 was identified. The loss of a consensus phosphorylation site as a result of this duplication was suggested to be important. The child's healthy parents were heterozygous for the mutation consistent with an autosomal recessive mode of inheritance [9].

Multiple deletion disorders

Most deletions of mtDNA are single and identical within an individual patient. This suggests that such deletions arise from a single deletional event early in development followed by clonal proliferation of the deleted molecule. The mechanism by which such single deletions form is not determined, but slippage during replication or recombination events have been suggested [86, 87, 89]. These single deletions are sporadic and are generally not transmitted to offspring. In contrast, multiple deletions are inherited as Mendelian traits, suggesting that they are due to defective nuclear genes [10]. Such patients harbour many different-sized delection (in contrast to the identical clonal delections in single-deletion cases), and the presumed nuclear defect is suggested to increase the propensity of mtDNA to form deletions. A large number of different phenotypes have been described in association with multiple deletions, the commonest being autosomal dominant CPEO syndrome. Linkage analysis in pedigrees with autosomal dominant CPEO has identified two loci, one on chromosome 10q23.3–24.3 and the other on chromosome 3p14.1–21.2 [109, 114]. Other families do not link to either of these loci, indicating further genetic heterogeneity [116]. Mitochondrial transcription factor A (mtTFA) represented a good candidate gene for multiple deletions but has been excluded [109].

mtDNA depletion syndrome

In this disorder there is a reduction in the total amount of mtDNA within tissues, often to extremely low levels.

The mtDNA is qualitatively normal. A number of different clinical phenotypes are described in association with mtDNA depletion, including a fatal infantile hepatopathy, a fatal infantile myopathy and a more benign infantile myopathy [88]. Cell fusion studies have provided support for the concept that this depletion syndrome is a nuclear genetic disorder [90]. One study showed that myoblast cultures, from a patient who died of hepatopathy, initially had normal levels of mtDNA, even though the patient's skeletal muscle from which the myoblasts were derived had marked depletion. However, the myoblasts became progressively depleted of mtDNA during culture, indicating that the depletion trait is only expressed after a certain stage in muscle development. This depletion was restored when the nuclear background was changed by fusion with a rho zero cell line [91]. Genetic complementation studies should facilitate the identification of the nuclear gene or genes responsible for the mtDNA depletion trait. mtTFA is essential for replication of mtDNA and is important in maintaining mtDNA copy number. It therefore represents a possible candidate gene for the depletion syndrome. However, recent studies in an mtTFA knockout mouse indicate that a reduction in mtTFA is unlikely to be the primary cause of tissue-specific mtDNA depletion in humans [92].

Isolated COX deficiency

One of the commonest causes of Leigh's syndrome is complex IV deficiency, and a large body of evidence indicates that in most cases this is a nuclear genetic disorder. Somatic cell genetic studies have established that a single major complementation group exists for most cases of COX-deficient Leigh's syndrome. This suggest that a single nuclear gene is responsible for most cases. All the nuclear-encoded subunits genes for COX have previously been cloned, and sequence studies have failed to identify pathogeneic mutations [93]. This has led to the suggestion that the nuclear gene responsible for COX-deficient Leigh's syndrome may code for a protein important for the assembly of the complex IV holoenzyme [11].

Respiratory chain dysfunction and common neurological disease

The respiratory chain disorders associated with defined defects of mtDNA or with nuclear gene defects are individually relatively rare. Much attention has recently focused upon the finding of respiratory chain dysfunction in two common neurological diseases, Huntington's disease and Parkinson's disease.

Huntington's disease

This is a common genetic neurodegenerative disorder characterised by the triad of psychiatric disturbance, a choreiform movement disorder and dementia. It is inherited in an autosomal dominant fashion, and a classical genetic linkage approach led to the discovery of the responsible gene in 1993 [94]. The genetic defect in the gene is an abnormally expanded trinucleotide repeat (CAG) which is translated into an expanded polyglutamine run in the gene product which is known as Huntingtin. The normal function of Huntingtin is not known. It is expressed widely in the CNS but also in other tissues outside the CNS which function normally in Huntington's disease. Neuropathologically the caudate nucleus of the basal ganglia is particularly affected. Disturbed respiratory chain function was first identified in the caudate and cerebral cortex by Brenan and coworkers [95]. Animal studies have lent support to the hypothesis that impaired respiratory chain function may be important in Huntington's disease. A specific complex II inhibitor, 3-nitropropionic acid, can reproduce much of the symptomatology and neuropathology [96]. Indirect excitotoxic cell death has been demonstrated to be the mechanism through which complex II inhibition operates in vitro [97].

Respiratory chain function in brain tissue from Huntington's disease patients has been analysed in two recent studies. In the first study caudate tissue was analysed, and a 53–59% decrease in complex II/III activity and a 32–38% reduction in complex IV activity were reported [98]. A second study investigated respiratory chain dysfunction noninvasively in eight Huntington's disease cases using the technique of magnetic resonance spectroscopy. All cases were demonstrated to have increased lactate concentrations in the occipital cortex, elevated lactate/pyruvate ratios in the cerebrospinal fluid and decreased phosphocreatine/inorganic phosphate ratios in resting skeletal muscle. These observations suggested a generalised defect of energy metabolism [99].

Parkinson's disease

Parkinson's disease is the commonest movement disorder, characterised by tremor, rigidity, akinesia and postural instability. Pathologically there is loss of dopaminergic neurons in part of the brainstem known as the substantia nigra. Dopamine replacement can ameliorate the symptoms for a period of time. Most cases are sporadic, but rare families with dominantly inherited Parkinson's disease are described. An anatomically specific complex I defect in the pars compacta was originally reported in 1989 and has subsequently been confirmed [100, 101]. The precise cause of this defect and its relevance to the pathogenesis of nigral cell death

remains to be determined. A number of studies have indicated that various mtDNA polymorphisms may be more common in Parkinson's disease patients than controls, but this has not been a consistent finding [102, 103]. The possibility that as yet unidentified polymorphisms within the mitochondrial genome are relevant to the observed complex I defect cannot be excluded, and cybrid studies have provided some evidence that this may indeed be the case. Using a cybrid system in which platelet-derived mitochondria from patients or controls were fused with neuroblastoma cells devoid of mitochondria, it has been shown that cybrids generated from patients with Parkinson's disease showed a stable 20% decrement in complex I activity compared with controls. This indicates that the decreased complex I activity is indeed secondary to defects in mtDNA [104]. To date no mtDNA sequence data from cybrids expressing this complex I defect has been reported.

Conclusions

A large number of primary defects of mtDNA associated with human disease have now been defined, and the elucidation of their precise molecular pathogenesis is progressing. Major unresolved issues include an understanding of the mechanisms involved in transmission and segregation of mtDNA mutants as well as the factors which influence the complex genotype-phenotype correlations observed. The development of animal models harbouring pathogenic defects in mtDNA is the next step. Nuclear gene defects leading to respiratory chain dysfunction are now starting to be defined, and it seems likely that many more will follow. Whilst respiratory chain dysfunction is clearly present in common disorders such as Huntington's disease and Parkinson's disease, the precise relationship this has to the pathogenesis of these disorders remains unresolved.

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